IS METHYLGLYOXAL A CAUSATIVE FACTOR FOR THE PATHOGENESIS OF TYPE 2 DIABETES MELLITUS AND ENDOTHELIAL DYSFUNCTION?

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By

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ABSTRACT

The number of people having diabetes mellitus is increasing worldwide at an alarming rate. An unbalanced diet rich in carbohydrates and saturated fats, obesity and lack of physical activity, are being blamed. The worldwide prevalence of diabetes for all age-groups has been estimated to be 2.8% in 2000 and projected to be 4.4% by the year 2030. The pathogenesis of diabetes, especially the recent epidemic increase in type 2 diabetes, is still far from clear. Endothelial dysfunction, commonly defined as reduced endothelium-dependent relaxation due to reduced availability of the vasodilator mediator nitric oxide (NO), is a hallmark of diabetes mellitus. Methylglyoxal (MG) is a highly reactive dicarbonyl compound mainly formed as an intermediate during glycolysis. MG is also formed to a lesser extent from protein and amino acid metabolism. However, the relative contribution of various metabolic precursors to MG formation is not known. Levels of MG have been found to be elevated in diabetic and hypertensive conditions but it is not known whether MG is the cause or the effect of these pathological conditions. The aim of my project was (i) to quantify the amount of MG and oxidative stress produced from various substrates in cultured A10 vascular smooth muscle cells (VSMCs), (ii) to investigate the acute *in vivo* effects of a single dose of MG on glucose tolerance in male Sprague-Dawley (SD) rats, (iii) to investigate the effects of MG on endothelial function and (iv) to investigate the effects, and the underlying molecular mechanisms, of chronic administration of MG on glucose homeostasis in male SD rats. The results show that aminoacetone, a protein metabolism intermediate, is the most potent substrate for MG formation on a molar basis, whereas D-glucose and fructose are equipotent. I also established optimum sample preparation protocols for reproducible

measurement of MG in biological samples by high performance liquid chromatography (HPLC). In normal SD rats a single acute dose of MG induced glucose intolerance, reduced adipose tissue glucose uptake and impaired the insulin signalling pathway, which was prevented by the MG scavenger and advanced glycation end product (AGE) breaking compound, alagebrium (ALT-711). MG and high glucose (25 mM) induced endothelial dysfunction in rat aortic rings and cultured endothelial cells by reducing endothelial nitric oxide synthase (eNOS) phosphorylation at Ser-1177, activity and NO production. MG and high glucose also increased oxidative stress and further reduced NO availability in rat aortic rings and cultured endothelial cells. Chronic administration of MG in normal SD rats by continuous infusion with a subcutaneously implanted minipump for 28 days (60 mg/kg/day), induced metabolic and biochemical abnormalities of glucose homeostasis and insulin regulation that are characteristic of type II diabetes. In MG treated rats, insulin stimulated glucose uptake in adipose tissue, and glucose stimulated insulin release from freshly isolated pancreas, were significantly reduced as compared to saline treated control rats. At a molecular level, insulin gene transcription was significantly impaired and apoptosis and DNA fragmentation were more prevalent in the pancreas of MG treated rats as compared to untreated control rats. All of these *in vivo* effects of MG were attenuated by the MG scavenger, alagebrium. Our data strongly indicate that MG is a causative factor in the pathogenesis of endothelial dysfunction and type 2 diabetes mellitus.

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In the beginning of my research carrier, I realized a researcher cannot complete a research project alone. Although the number of people I want to thank goes beyond the format of thesis, I would like to especially thank the following people for their guidance, support and encouragement.

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Last but not least, there are no words to pay obeisances to God.

DEDICATION

To my parents

Sh. Moti Lal Dhar and Smt. Usha Dhar

For their constant encouragement, never ending love, care and support

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Abbreviations

2-MQ	2-methyl quinoxaline
3-DG	3-Deoxyglucosone
5-MQ	5-methyl quinoxaline
AG	Aminoguanidine
AGEs	Advanced glycation end products
ALA	Alagebrium
AMO	Acetol monooxygenase
ANOVA	One way analysis of variance
CEL	Nε-carboxy ethyl lysine
CML	Nε-carboxy methyl lysine
DHAP	Dihydroxyacetone phosphate
DM	Diabetes mellitus
DMEM	Dulbeccos'modified eagle medium
EC	Endothelial cells
eNOS	Endothelial nitric oxide synthase
F12 media	Kaighns F12 medium
G-3-P	Glyceraldehyde-3-phosphate
GLUT	Glucose transporter
GSH	Reduced glutathione
GSH-Red	Glutathione reductase
GTT	Glucose tolerance test

H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
HUVECs	Human umbilical vein endothelial cells
IDDM	Insulin dependent diabetes mellitus
iNOS	Inducible nitric oxide synthase
InsR	Insulin resistance
IR	Insulin receptor
IRS-1	Insulin receptor substrate 1
ITT	Insulin tolerance test
MG	Methylglyoxal
NAC	N-acetyl L-cysteine
NO	Nitric oxide
$O_2^{\cdot -}$	Superoxide
$ONOO^-$	Peroxynitrite
o-PD	Ortho phenylene diamine
PBS	Phosphate buffered saline
PCA	Perchloric acid
PI3K	Phosphoinositide 3 kinsase
RAECs	Rat aortic endothelial cells
ROS	Reactive oxygen species
SD	Sprague-Dawley
SSAO	Semicarbazide-sensitive amine oxidase
VSMCs	Vascular smooth muscle cells

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Diabetes mellitus

Diabetes mellitus (DM), as defined in Harrison's Principles of Internal Medicine, is "a group of common metabolic disorders that share the phenotype of hyperglycemia" (page-2275). DM is usually characterized by abnormally high blood glucose levels (hyperglycemia), glycosuria (glucose in urine), hyperlipidemia and sometimes ketonemia. Because of the constant exposure to high concentrations of glucose, there is increased nonenzymatic glycosylation of proteins which is considered to be a causative factor in the pathological changes of diabetes (Harrison's Principles of Internal Medicine). As DM progresses, there is increased thickening of capillary basement membrane, and increased cellular proliferation, which result in vascular complications such as atherosclerosis, retinopathy, neuropathy and peripheral vascular insufficiency. Insulin enables the cells to absorb glucose and convert it into energy. In case of DM, there is either a deficiency of insulin or high insulin levels due to the body's inability to properly respond to insulin to meet its needs. This phenomenon creates a vicious cycle resulting in persistent hyperglycemia, often leading to various complications (Rother, 2007). Maintaining optimum glucose control minimizes the risk of long term complications of diabetes.

In 2008, there were 1.66 million people with diabetes in Canada compared to 1.22 million in 2003, a staggering increase of 36% in 5 years (Diabetes, by sex, provinces and territories). In the year 2009, the American Diabetes Association reported that 23.6 million children and adults in the United States, i.e., 7.8% of the population, had diabetes. It is estimated that 17.9 million in the US alone have been diagnosed with diabetes, and there are another 5.7 million (nearly one in four) diabetics who are not even aware that they have the disease (Diabetes facts, Centers for Disease Control, USA). The World Health Organization

had predicted that by 2010, there will be more than 230 million people diagnosed with diabetes world-wide out of which three million will be in Canada (Diabetes Report, Diabetes 2000; Diabetes. The Provincial Plan). The risk of heart disease or stroke is six times and the risk of high blood pressure is four times higher for people with diabetes aged 35-64 years, compared to the general population. In Canada the total costs of health care and services, and loss of work resulting from diabetes is estimated to be $ 9 billion annually (Diabetes Report, Diabetes 2000; Diabetes. The Provincial Plan).

	Normoglycemia	Hyperglycemia		
Types of diabetes		Prediabetes	Diabetes	
	Normal glucose tolerance	Impaired fasting glucose or impaired glucose tolerance	Not insulin requiring	Insulin required for control	Insulin required for survival
Type 1	←	—	—	—	→
Type 2	←	—	—	→	
Gestational	←	—	—	→	
Other types	←	—	—	→	
Time (years)	→				
Fasting plasma glucose	<5.6 mmol/L (100 mg/dL)	5.6-6.9 mmol/L (100-125 mg/dL)	≥7.0 mmol/L (126 mg/dL)		
2h plasma glucose	<7.8 mmol/L (140 mg/dL)	7.8–11.1 mmol/L (140-199 mg/dL)	≥11.1 mmol/L (200 mg/dL)		

Table 1-1. Main types of diabetes mellitus and the associated plasma glucose levels and insulin-dependency across the life span (Adapted from Harrisons Principles of Internal Medicine, 17th edition, 2008)

There are several types of diabetes mellitus (American Diabetes Association, 2007) with distinct differences which arise from complicated interactions between genetic and environmental factors. An extensive etiologic classification has been formulated (American Diabetes Association, 2007). The three main types of diabetes mellitus (Table 1) are described below:

1.1.a Type I diabetes mellitus

Type I diabetes mellitus (T1DM) also used to be known as insulin dependent diabetes mellitus (IDDM) or juvenile onset diabetes mellitus, terms that are no longer used. In T1DM, there is pancreatic β cell destruction and patients have to depend on insulin injections for survival. The majority of cases of T1DM are due to autoimmunity. In T1DM, the immune system mistakenly manufactures antibodies and inflammatory cells which cause damage to the patient's own body (Cooke and Plotnick, 2008). The most commonly observed antibodies in T1DM are anti-islet cell antibodies, anti-insulin antibodies and anti-glutamic decarboxylase antibodies. These antibodies are used as markers to identify individuals at a risk of developing T1DM. However some cases of T1DM are idiopathic, that is no β cell antibodies are found (Gillespie, 2006). The abnormal antibodies found in T1DM are in part genetically inherited but the details are not yet fully known (Van den Driessche *et al*., 2009). The number of patients having T1DM is ~10% whereas the remaining 90% have type II diabetes mellitus (T2DM), which is described later (Gillespie, 2006).

There are a number of animal models used for T1DM research. The non obese diabetic (NOD) mouse develops diabetes spontaneously (Makino *et al*., 1980) and is often used as a T1DM model. Another animal model often used in research laboratories is the streptozotocin

(STZ)-induced diabetes. STZ is a chemical which causes destruction of β cells of the pancreas and produces symptoms similar to T1DM (Cefalu, 2006).

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1.1.b Type 2 diabetes mellitus

T2DM also used to be known as non-insulin dependent diabetes mellitus (NIDDM) or maturity onset diabetes mellitus as it usually occurs in people above the age of 30 (Lebovitz, 2001). The latter terms are no longer accepted. T2DM is characterized by insulin resistance, impaired β cell function and glucose regulation which ultimately lead to β cell failure. In the case of T2DM, there are insulin producing functional β cells in varying numbers, despite gradual progressive apoptosis in the advanced stages of the condition. In the early stage of T2DM, the insulin resistance, characterized by defects in the insulin signalling pathway (Reaven, 1988), elicits a compensatory increase in pancreatic insulin secretion and higher than normal plasma insulin levels (Fig. 1). However, as the disease progresses, the release of insulin from the pancreas becomes reduced, due to apoptosis and decrease in β cell numbers in the later stages of T2DM, resulting in lower than normal plasma insulin levels and hyperglycemia. The apoptosis of pancreatic islet β cells in T2DM differentiates it from the necrosis of β cells seen in T1DM. Thus, insulin in the circulation can be either high or low depending on the stage of diabetes (Fig. 1). Poor eating habits, higher body weight and lack of physical exercise make people more susceptible to develop T2DM (Israili, 2009; Barnett, 2009). T2DM has a strong genetic predisposition, however there are other risk factors involved, the most notable of which is obesity. Previous studies have reported that the risk of developing T2DM doubles for every 20% increase in the body weight (Inzucchi and Sherwin, 2005).

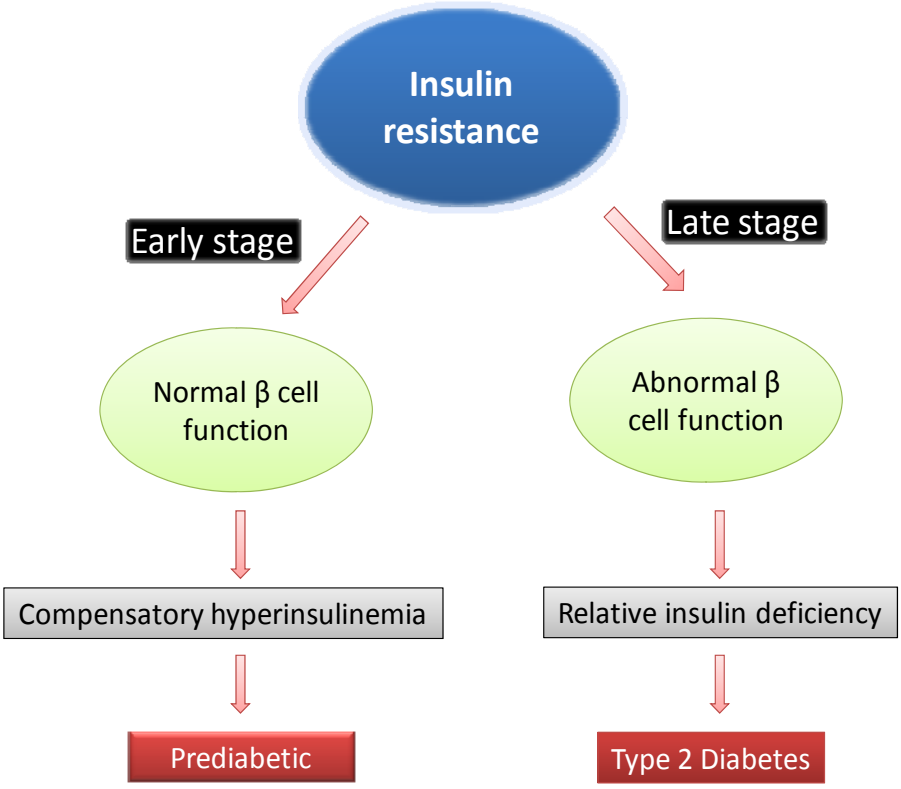

Figure 1-1. The relationship between pancreatic β cell function, plasma insulin levels and the stages of type 2 diabetes mellitus.

Glucose plays a key role in insulin secretion from the pancreatic β cell. Insulin secretion starts with glucose transport by the glucose transporter 2 (GLUT2) in pancreas which in turn induces an increase in glucokinase. Glucokinase induces phosphorylation of glucose by glycolysis leading to the generation of adenosine triphosphate (ATP) which inhibits the ATP sensitive potassium (K_{ATP}) channels, causing hyperpolarization, and increasing intracellular calcium which ultimately leads to exocytosis of the insulin granules. In the case of T2DM, glucose transport is greatly reduced (Porte, 1991). As the disease progresses, the release of newly synthesized insulin is impaired. This phenomenon is termed as 'desensitization' or 'β

cell toxicity' (Malaisse, 1996). In some families of T2DM patients an absence of glucokinase has been reported (Nicholls *et al*., 1995), however, this has not been seen in other families of T2DM. In the majority of cases of T2DM, there is an inherited or acquired defect within the β cell. As the disease progresses there is a decrease in insulin secretion due to accumulation of glucose metabolites (Mahler and Adler, 1999).

As described by Neubauer and Kulkarni (2006), there are a number of animal models of T2DM like the ob/ob (obese) or db/db (diabetes) mice. The ob/ob mouse model was created by inducing mutation in the leptin gene whereas the db/db model was created by inducing mutation in the leptin receptor. Leptin is a hormone secreted mainly by the adipocytes in response to fatty acid storage in these cells. Leptin plays a negative feedback role in food intake, energy production, and weight regulation. Defects in leptin production or leptin receptor disrupt this feedback cycle and cause weight gain and obesity (Neubauer and Kulkarni, 2006). The Zucker diabetic fatty (ZDF) rat is another T2DM animal model widely used in research. ZDF rats also have mutation in their leptin receptor and become obese within the first few months of life (Clark *et al*., 1983; Peterson *et al*., 1990). The Goto Kakizaki (GK) rat is a model of T2DM with the characteristic that it is non obese and has decreased β cell mass (Goto and Kakizaki, 1981). As diet contributes to the development of diabetes, a diet high in fat or carbohydrate accelerates the development of T2DM (Cefalu, 2006). One such model of diet-induced diabetes is the fructose-induced diabetes in Sprague-Dawley (SD) rats (Hwang *et al*., 1987). Our lab has shown that SD rats fed high fructose (60% of total dietary calories) develop insulin resistance (Jia and Wu, 2007). The use of an appropriate animal model can provide insights into the pathophysiological mechanisms and molecular targets for the development of effective and safer drugs and prevention of the disease. T2DM differs

from T1DM in many respects as shown in table 2.

Table 1-2. Differences between type 1 and type 2 diabetes mellitus

Type 1 Diabetes	**Type 2 Diabetes**
Prevalence less than 10%	Prevalence more than 90%
Onset: typically in childhood Symptoms usually start in childhood or young adult	Onset: typically middle-age and later Recently many childhood onset cases May not have symptoms before diagnosis
Genetic predisposition moderate Autoimmune disease	Genetic predisposition is very strong
No or very little insulin	Combination of insulin resistance and insulin secretary defect
Episodes of low blood sugar level (hypoglycaemia) common with insulin treatment	Hyperglycemia mostly, episodes of low blood sugar level relatively few
Cannot be prevented	Can be prevented or delayed with healthy life style
No glucose intolerance	Glucose intolerance
Ketonemia and ketoacidosis common with uncontrolled hyperglycemia	Not commonly seen

1.1.c Gestational diabetes mellitus

Gestational diabetes occurs in pregnant women who have high blood glucose levels

during pregnancy. Gestational diabetes may lead to T2DM or sometimes T1DM in predisposed people, but is usually resolved once the baby is born. Oral glucose tolerance test is done after delivery to determine if the patient is at a future risk of developing diabetes. 4% of all pregnant women are affected by gestational diabetes (Diabetes Care, 2004).

1.1.d Insulin signalling pathways

The hormone insulin is secreted by the β cells of the pancreatic islets in response to several stimuli, the main one being an elevation of blood glucose concentration. Insulin has anabolic functions. Thus, it helps in growth and development and maintenance of glucose homeostasis. Insulin plays a major role in glucose homeostasis, glycogen and protein synthesis, the inhibition of lipolysis and the stimulation of fat storage (Saltiel and Kahn, 2001; Saltiel and Pessin, 2002). The insulin receptor is a tyrosine kinase receptor located in the cell plasma membrane (Fig. 2). Stimulation of the insulin receptor phosphorylates and activates proteins such as Cb1 (Saltiel and Kahn, 2001), APS [an adaptor protein (Moodie *et al*., 1999)], Shc (an adaptor protein), Gab-1 [Grb-2 associated binding protein 1 (Holgado-Madruga *et al*., 1996)] and insulin receptor substrate (IRS) proteins on tyrosine residues (Fig. 2). The downstream effectors are components of three major pathways: the more insulin specific phosphatidylinositol-3 kinase (PI3K) and the Cb1, and the more common mitogen activated protein kinase (MAPK) pathway, through which many growth factors signal. Recruitment of PI3K by the activated IRS proteins results in conversion of membrane lipid phosphatidylinositol (Jia & Wu, 2007) bisphosphate (PIP_2) to phosphatidylinositol (Jia & Wu, 2007) trisphosphate (PIP_3). PIP3 activates phosphoinositide-dependent kinase-1 (PDK1), which in turn activates protein kinase (PKB, also known as Akt). Activation of Cb1 causes

GLUT4 translocation from the cytosolic vesicles to the cell plasma membrane to facilitate glucose transport into the cell (Fig. 1-2). Insulin reduces hepatic glucose production by decreasing gluconeogenesis and glycogenolysis, and it promotes glucose uptake into insulin-sensitive tissues such as skeletal muscle and adipose tissue.

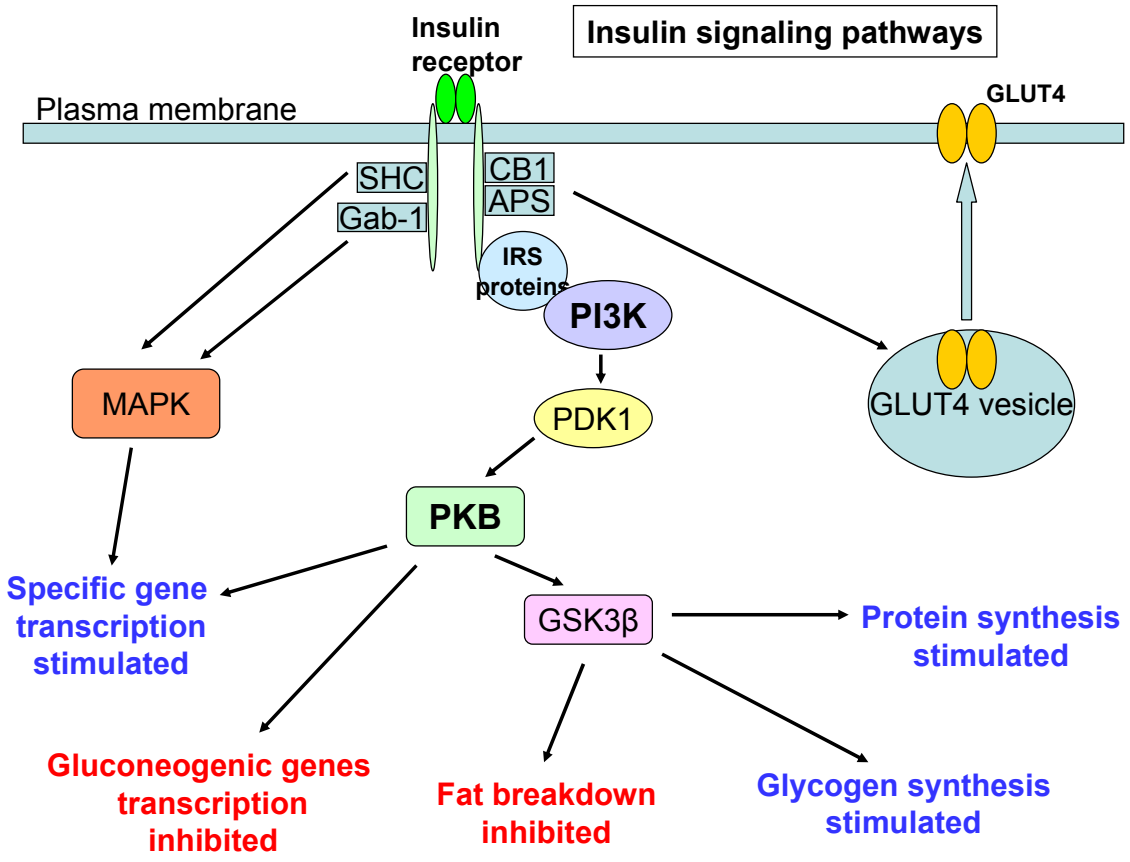

Figure 1-2. Insulin's metabolic and growth promoting signalling pathways. The schematic shows the major components in simplified signal transduction pathways stimulated by insulin acting on the insulin receptor.

Abbreviations: Glycogen synthase kinase 3β (GSK3β), protein kinase B (PKB), phosphoinositide-dependent kinase-1 (PDK1), mitogen activated protein kinase (MAPK),

insulin receptor substrate (IRS), glucose transporter 4 (GLUT4), phosphatidylinositol-3 kinase (PI3K).

1.1.e Insulin gene transcription

The β cells of pancreas contain a large pool of insulin mRNA which accounts for about 10-15% of total β cell mRNA. At low plasma glucose concentration, the insulin mRNA is due to basal gene transcription but as the plasma glucose concentration increases, the insulin mRNA increases 3-4 fold and the effect is most pronounced 30 min after glucose stimulation and declines thereafter (Leibowitz *et al*., 2001). The regulation of insulin mRNA production is complex and a number of factors are involved in gene expression. There are a number of factors involved in insulin gene transcription among which are pancreatic and duodenal homeobox-1 (PDX-1), Maf and a heterodimer with a main glucose responsive element on the insulin promoter, A3 (Fig. 1-3). PDX-1, also known as insulin promoter factor 1 (IPF-1), is a transcription factor involved in β cell maturation and insulin secretion. Previous studies in Psammomys obesus, a model of T2DM have shown an apparent lack of PDX-1 in the pancreas, which is the main mediator of insulin gene expression and pancreas development (Leibowitz *et al*., 2001). Glucose stimulation of the pancreas shifts PDX-1 from the cytoplasm to the nucleus and increases binding of PDX-1 to A3 (Fig. 3). Glucose and PDX-1 also regulate insulin gene transcription by influencing histones. Under low glucose conditions, PDX-1 interacts with histone deacetylase Hdac-1 and Hdac-2, recruits them to the insulin gene promoter, deacetylate histone H4 and thus downregulates insulin gene expression (Ren *et al*., 2007; Leibowitz *et al*., 2001).

Another transcription factor involved in insulin secretion is Maf-A (Fig. 1-3). Maf-A is

a basic leucine zipper transcription factor and it regulates gene expression in the pancreas. Maf-A is mainly expressed in β cells of the islets of Langerhans and is involved in insulin gene transcription, insulin secretion and β cell survival (Vanderford *et al*., 2007). Maf-A is activated by glucose by a distinct mechanism. At low glucose concentration, Maf-A is almost undetectable in β cells but as the glucose concentration increases, Maf-A protein is rapidly detected. With increasing duration of exposure to high concentration of glucose, Maf-A mRNA also increases significantly. Maf-A knockout mice develop glucose intolerance, impaired glucose stimulated insulin release, pancreatic islet abnormalities and diabetes mellitus (Zhang *et al*., 2005).

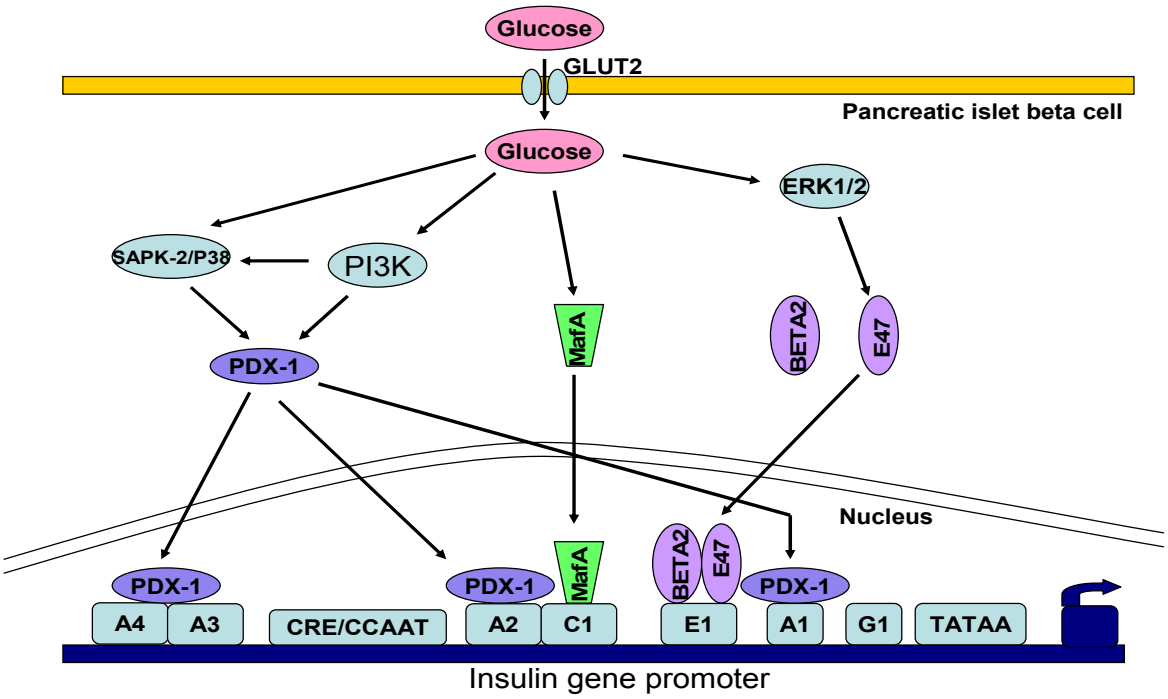

Figure 1-3. Schematic representation of the proximal region of the rat insulin promoter showing the key cis-acting DNA elements and trans-activating factors involved in glucose regulation of insulin gene transcription.

CCAAT-enhancer binding protein (C/EBPβ) is a negative regulator of insulin gene expression (Plaisance *et al*., 2009). It stimulates gluconeogenesis in the liver and adipogenesis in the adipose tissue. In db/db mice knockdown of C/EBPβ reduces diabetes, besides reducing fatty liver disease and obesity. It was proposed as a novel target for the treatment of fatty liver disease and obesity by the authors (Schroeder-Gloeckler *et al*., 2007).

1.1.f Insulin resistance

Insulin resistance can be broadly defined as decreased responsiveness of target tissues to normal circulating levels of insulin. Besides playing a role in the development of T2DM, insulin resistance is also a feature of the metabolic syndrome, which also involves hypertension, dyslipidemia, obesity and glucose intolerance (Sechi & Bartoli, 1997).

Studies to determine the pathogenesis of insulin resistance have shown that defective muscle glycogen synthesis plays a major role because glycogen synthesis in the skeletal muscle (Bogardus *et al*., 1984; Kelly *et al*., 1996; Damsbo *et al*., 1991), a main target of insulin-mediated glucose uptake, is the major pathway for glucose metabolism in normal and T2DM patients (Shulman *et al*., 1990). The liver also shows reduced glycogen stores in insulin-resistant diabetic subjects (Damsbo *et al*., 1991).

Insulin-stimulated phosphorylation of the insulin receptor has been shown to be impaired in skeletal muscle of T2DM patients (Arner *et al*., 1987), and obese people (Goodyear *et al*., 1995), in adipose tissue of obese subjects with and without T2DM (Sinha *et al*., 1987), and in liver of T2DM patients (Caro *et al*., 1986).

Similarly, decreased phosphorylation of IRS-1 and PI 3-kinase in response to insulin

has been observed in skeletal muscle and adipocytes of obese people and T2DM patients (Goodyear *et al*., 1995; Bjornholm *et al*., 1997; Smith *et al*., 1999). Insulin-stimulated phosphorylation of Akt (protein kinase B, PKB) has been shown to be decreased in skeletal muscle in T2DM patients (Brozinick *et al*., 2003; Krook *et al*., 1998).

1.2 Endothelial dysfunction

Endothelial cells form the inner lining of blood vessels. The endothelial cells have been widely studied and it is now recognized that they perform a wide variety of functions. Some of the functions of the endothelial cells include regulation of vasodilation/vasoconstriction, coagulation/anticoagulation, vascular smooth muscle cell proliferation, angiogenesis, capillary permeability, vascular immune responses and inflammation. Broadly speaking endothelial dysfunction can be taken to mean a disruption of any one or more of these functions. The role of the endothelium in vascular relaxation was discovered by the seminal observations of Professor Robert Furchgott (Furchgott and Zawadzki, 1980) who elegantly demonstrated that acetylcholine (ACh) induced vascular smooth muscle relaxation by stimulating the release of a relaxing substance from the endothelium, which he called "endothelium-derived relaxing factor" or EDRF. EDRF was later identified as nitric oxide (NO) (Palmer *et al*., 1987). Since the discovery of EDRF by Furchgott, endothelial dysfunction has been typically described as reduced endothelium-dependent relaxation. More strictly, most studies of endothelial dysfunction refer to reduced availability of NO (Fig. 1-4). This notion is being challenged by studies reporting reduced availability of other endothelium-derived mediators that cause relaxation such as endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin in certain vessels (Chen *et al*., 1988;

Taylor and Weston, 1988; Hecker *et al*., 1994; Chauhan *et al*., 2003; Bolotina *et al*., 1994; Whittle *et al*., 1978). The discovery of hydrogen sulphide (Wang, 2009) and carbon monoxide (Mustafa *et al*., 2009) as endothelium derived relaxants will complicate the issue further.

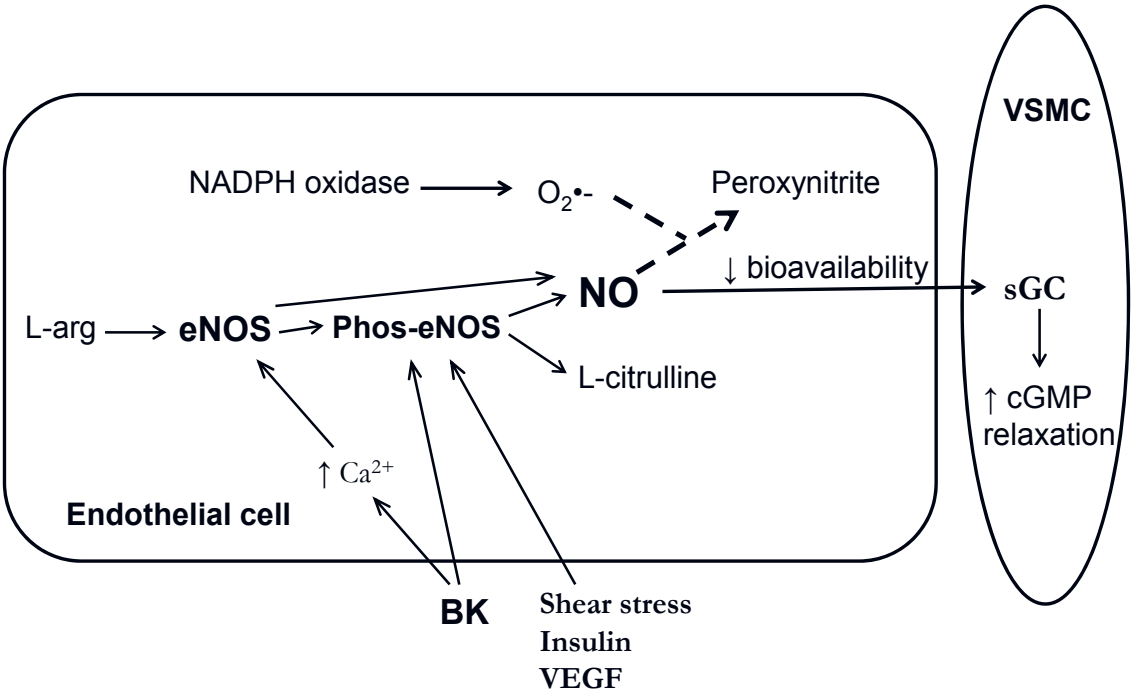

Figure 1-4. Nitric oxide mediated endothelium-dependent relaxation and endothelial dysfunction.

Schematic figure shows an endothelial cell and the adjacent vascular smooth muscle cell (VSMC). Stimuli such as bradykinin (BK), shear stress, insulin and vascular endothelial growth factor (VEGF) activate endothelial nitric oxide synthase (eNOS) by causing an increase in intracellular calcium (Ca^{2+}) or by causing phosphorylation of eNOS (Phos-eNOS), and produce nitric oxide (NO) from the substrate L-arginine (L-arg). NO diffuses to the VSMC, where it activates soluble guanylate cyclase (sGC), increases cyclic guanosine

monophosphate (cGMP) and causes relaxation (Evora and Nobre, 1999). Under conditions of oxidative stress, an increased superoxide anion (O_2•-) is produced, mainly from NADPH oxidase, which reacts with NO and produces peroxynitrite. This reduces the bioavailability of NO and causes endothelial dysfunction. Reduced activity of eNOS can also cause reduced NO production (Endemann and Schiffrin, 2004)**.** This figure does not take into account altered production of other vasodilators such as EDHF and prostacyclin, or vasoconstrictors such as endothelin, that may be responsible for dysfunction.

Endothelial dysfunction is a hallmark of diabetes (Escandon and Cipolla**,** 2001; Sowers, 2002), atherosclerosis and hypertension (Davignon and Ganz, 2004), and it is associated with numerous other conditions. It is characterized by reduced endothelium dependent vasorelaxation, a proinflammatory and prothrombic state (Endemann and Schiffrin, 2004). The pathophysiology of endothelial dysfunction is complex and involves multiple mechanisms, however some of these seem to be common to most conditions. The most important vasodilating substance released by endothelium is NO, which functions as a vasodilator, inhibits growth of vascular smooth muscle, inflammation and has anti-aggregatory action on platelets. As mentioned earlier, reduced NO has often been reported in conditions of endothelial dysfunction. There are a number of reasons for this, such as reduced availability of co-factor tetrahydrobiopterin (Consentino *et al*., 2008), reduced activity of endothelial nitric oxide synthase (eNOS) due to endogenous (Boger *et al*., 2000; Jang *et al*., 2000) or exogenous inhibitors (Chiesi and Schwaller, 1995), reduced availability of its substrate L-arginine (Lekakis *et al*., 2002) or rapid destruction of NO by superoxide anion (Ma *et al*., 2001). Reactive oxygen species (ROS), especially superoxide, are often known to quench NO, forming peroxynitrite, a reactive cytotoxic oxidant. Peroxynitrite causes nitration

of proteins, affects protein function and therefore endothelial function (Koppenol *et al*., 1992). In animal models of hypertension, oxidative stress has been shown to induce endothelial dysfunction and the improvement of endothelium-dependent vasorelaxation after administration of antioxidants (Chen *et al*., 2001). The most commonly used parameter for assessing endothelial function is endothelium-dependent vasodilation. It is performed angiographically in coronary arteries by laser Doppler flow measurement by assessing the effect of endothelium-dependent agonists like ACh (Schachinger *et al*., 2000). Cold pressor test is another way of assessing endothelial function. It involves the measurement of coronary perfusion by positron emission tomography scanning as a measure of endothelial function (Schindler *et al*., 2003). Endothelial function can also be assessed on forearm resistance arteries by forearm blood flow measurement using strain gauge plethysmography (Perticone *et al*., 2001). In animal models endothelial function is measured as changes in endothelium-dependent relaxation in isolated aortic rings, mesenteric artery or one of the other resistance arteries.

1.2.a Mediators released from endothelium

Nitric oxide:

NO is a vasodilator substance with a short half life of about 5-10 seconds. NO can be differentiated from prostacyclin, one of the other relaxant mediators released by the endothelium, by its ability to be un-affected by cyclooxygenase (COX) inhibitors. EDRF was first discovered by Robert Furchgott. Furchgott in his study showed that endothelial lining is important for vasorelaxant action of ACh on aortic rings or arterial strips isolated from rabbit. Ach acts on muscarinic receptors on the surface of endothelial cells, generates NO, which

diffuses to the vascular smooth muscle cells, activates soluble guanylyl cyclase (sGC), which converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) and causes relaxation. There are also other vasorelaxant substances released by endothelium like prostacyclin, carbon monoxide and endothelium derived hyperpolarising factor (EDHF). Besides ACh, some of the other substances which generate NO are bradykinin, histamine, calcium ionophore, norepinephrine and 5-hydroxytryptamine (Furchgott, 1996; Brandes *et al*., 2000).

Endothelium-derived hyperpolarizing factor

After the discovery of EDRF in and NO in the 1980s it was recognized that ACh caused vascular relaxation which was not prevented by inhibitors of NO and prostacyclin production, and which was caused by hyperpolarization of the smooth muscle (Chen *et al*., 1988). A diffusible mediator released by the endothelium was proposed as endothelium-derived hyperpolarizing factor by Weston (Taylor and Weston, 1988; Komori and Vanhoutte, 1990). EDHF typically open potassium channels to cause hyperpolarization and vascular smooth muscle relaxation. Subsequently, extensive research has been done to establish the chemical identity of the EDHF. Many putative substances have been discovered and proposed as EDHF. These include a cytochrome P450 metabolite of arachidonic acid (Hecker *et al*., 1994; Fisslthaler *et al*., 1999), epoxyeicosatrienoic acid (EET) (Campbell *et al*., 1996); NO itself (Bolotina *et al*., 1994; Chauhan *et al*., 2003), hydrogen peroxide (Matoba *et al*., 2000), C-type natriuretic peptide (Chauhan *et al*., 2003; Villar *et al*., 2007; Ahluwalia an Hobbs, 2005), heterocellular gap junctions (Chaytor *et al*., 1998; Yamamoto *et al*., 1998; Hutcheson *et al*., 1999), K+ ion itself (Edwards, *et al*., 1998). EDHF plays a compensatory and key role in

the absence of NO and prostacyclin (Brandes *et al*., 2000; Desai *et al*., 2006; Scotland *et al*., 2005).

Prostacyclin

Prostacyclin is also known as prostaglandin I_2 (PGI_2) and it is a member of a family of molecules known as eicosanoids. PGI_2 is produced by the vascular endothelial cells and is a vasodilator and inhibits platelet aggregation (Whittle *et al*., 1978; Coleman *et al*., 1994; Praticò and Dogne, 2009; Gleim *et al*., 2009). PGI_2 has a short half life of about seconds and is converted into 6-keto PGF_1 which has less vasodilator property than PGI_2 (Smyth and FitzGerald, 2002). Prostacyclin plays an important role as vasodilator in some vascular beds such as the coronary circulation (Duffy *et al*., 1999) and skeletal muscle arterioles (Sun *et al*., 2006). There are a number of synthetic prostacyclin analogues available in the market like iloprost, cicaprost. They are mainly used as vasodilators in pulmonary hypertension or ischemia (Aronoff *et al*., 2007; Kermode *et al*., 1991).

Hydrogen sulphide

Hydrogen sulphide (H_2S) is another gaseous vasorelaxant produced by the endothelial cells (Wang, 2009). H_2S is produced from L-cysteine by the enzymes cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) (Wang, 2009). CSE knockout mice have high blood pressure and muscarinic stimulation of the endothelial cells produces H_2S mediated relaxation by activation of K_{ATP} channel and hyperpolarization of the vascular smooth muscle cells (Yang *et al*., 2008).

Carbon monoxide

Carbon monoxide (CO) is the third gaseous vasorelaxant produced by the endothelial cells (Mustafa *et al*., 2009). It is produced from the degradation of heme by heme oxygenase 2. CO activates soluble guanylyl cyclase and increases cGMP levels to produce vascular smooth muscle relaxation like NO (Wu and Wang, 2005). However, the function of CO as a vasorelaxant is still not clear (Mustafa *et al*., 2009).

Endothelin

Endothelin (ET) is produced by endothelial cells and is involved in the regulation of vascular function (Yanagisawa *et al*., 1988; Agapitov and Haynes, 2002). ET is a peptide and a very potent vasoconstrictor. Under normal physiological conditions, there is a fine balance between NO, which is a vasodilator and ET, however when the balance is disturbed, it leads to endothelial dysfunction (ED). Three isoforms ET1, ET2, ET3 and two receptors ET_A and ET_B have been identified (Hynynen and Khalil, 2006). These three isoforms are 21 amino acid peptides and have varying tissue distribution. ET acts on ET_A receptors on vascular smooth muscle and induces vasoconstriction. ET also acts on ET_B receptors on the surface of endothelial cells and induces vasodilation (Barnes and Turner, 1997). Thus ET induces both vasoconstriction and vasodilation. Intravenous injection of ET in animals causes an increase in blood pressure. Since ET is a powerful vasoconstrictor and causes increase in blood pressure, a number of ET antagonists have been discovered and are used in therapeutics. The first non-peptide, orally effective ET receptor antagonist discovered was bosentan. Bosentan has been widely used both in basic and clinical research (Rubin *et al*., 2002). The Food and Drug Administration (FDA) of USA has approved clinical use of bosentan for the treatment of

pulmonary hypertension.

1.2.b Regulation of endothelial nitric oxide synthase

NO is produced by the enzyme nitric oxide synthase (NOS) from the substrate L-arginine (Palmer *et al*., 1988). There are three known isoforms of NOS *viz*. endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) (Alderton *et al*., 2001). eNOS and nNOS are constitutive whereas iNOS is induced in cells such as macrophages and vascular smooth muscle cells by stimuli as cytokines, bacterial lipopolysaccharides (LPS) etc (Alderton *et al*., 2001). eNOS and nNOS require an elevation of intracellular calcium for activation whereas iNOS is calcium-independent for its activity. eNOS is primarily expressed in endothelial cells, nNOS in neuronal cells and iNOS in macrophages, VSMCs and other cell types (Alderton *et al*., 2001). eNOS has been extensively studied in relation to its regulation. eNOS is membrane associated, primarily with the golgi (Sessa *et al*., 1995) and also with plasma membrane caveoli (Garcia-Cardena *et al*., 1996; Feron *et al*., 1996; Fulton *et al*., 2002). Several regulatory proteins have been discovered which regulate its expression and activity at translational and posttranslational level. In endothelial cells NO is continuously produced by fluid shear stress and pulsatile stretch caused by blood flow (Corson *et al*., 1996; Fleming *et al*., 1998; Dimmeler *et al*., 1999), which does not depend on intracellular calcium (Fleming *et al*., 1998) . An increase is intracellular calcium in response to agonists such as ACh and bradykinin (Fleming *et al*., 2001) is another major mechanism for activation of eNOS. However, the binding of calmodulin to the eNOS enzyme is determined by phosphorylation and dephosphorylation of eNOS enzyme at specific amino acid residues, which include serine 1177 and threonine 495

(Fleming *et al*., 2001; Fulton *et al*., 1999; Dimmeler *et al*., 1999; Fleming 2010). A number of kinases and phosphatases also take part in this process of eNOS regulation and activity (Fleimng *et al*., 2001; Fleming, 2010). Several research papers have been published using endothelial cells from different species using different agonists to stimulate eNOS activity and NO production and investigate the regulatory pathways (Fulton *et al*., 1999; Fleming *et al*., 1998; Bernier *et al*., 2000; Fleming *et al*., 2001; Davda *et al*., 1994; Drummond *et al*., 2000; Haynes *et al*., 2000). As a result an integrated regulatory pathway involving key agonists, phosphorylation dephosphorylation status, calcium-dependency, phosphatases and kinases is difficult to put into place.

1.3 Methylglyoxal

Methylglyoxal (MG) is an aldehyde compound with two carbonyl groups (Fig. 1-5). Chemically it is also known as pyruvaldehyde, pyruvic aldehyde or 2-oxopropanal. It is a yellow liquid with a pungent odour. MG has a low molecular weight of 72.06.

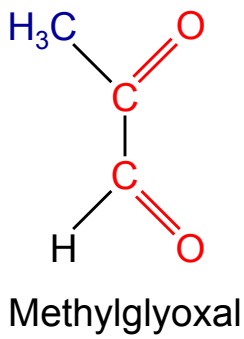

Figure 1-5. Chemical structure of methylglyoxal

1.3.a Synthesis of MG

Von Penchmann (1887) first developed the method for the synthesis of MG. In his study he showed that iso-nitrosoacetone heated in the presence of dilute sulfuric acid forms MG. However, along with MG other byproducts such as glycols, formaldehyde and formate were also formed. MG can be directly formed from methylglyoxal dimethylacetal by acid hydrolysis and also from oxidation of acetone with selenium dioxide followed by distillation under nitrogen atmosphere (Kalapos, 1999).

In living organisms, MG is formed from several metabolic pathways (Fig. 1-6). The most common and important source of MG formation is glucose metabolism. During glycolysis, MG arises from non-enzymatic elimination of phosphate from glyceraldehyde-3-phosphate (G-3-P) and dihydroxyacetone phosphate (DHAP) (Thornalley, 1996) (Fig. 1-6). Enzymatic formation of MG may involve leakage from active site bound phosphoene-diolate on triose phosphate isomerase.

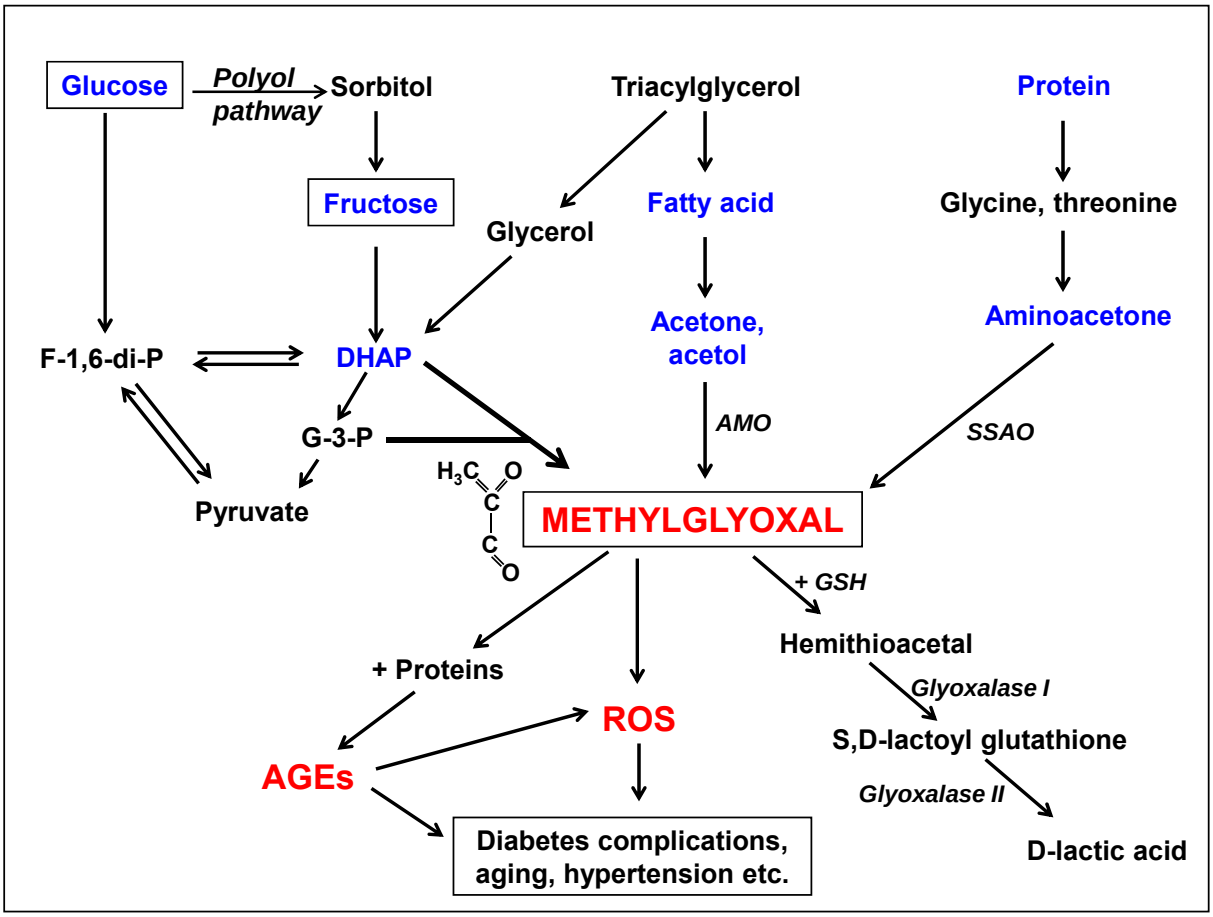

Figure 1-6. Metabolic pathways of MG formation from different substrates

Schematic showing pathways, substrates and enzymes involved in the formation of MG in the body. Glucose is the principal substrate.

Abbreviations: AGEs – advanced glycation endproducts; AMO – acetol/acetone monooxygenase; DHAP – dihydroxacetone phosphate; F-1,6-di-P – fructose-1,6-diphosphate; G-3-P – glyceraldehyde-3-phosphate; GSH – reduced glutathione; SSAO – semicarbazide-sensitive amine oxidase; ROS – reactive oxygen species.

[*Adapted from Metabolism. 2008 Sep; 57(9):1211-20.*]

MG can also be formed by the enzyme semicarbazide-sensitive amine oxidase (SSAO)

from aminoacetone, which is an intermediate of threonine catabolism (Fig. 6) (Yu, 2003). Another source of MG is fatty acid metabolism. In this case the enzyme acetol monooxygenase (AMO) forms MG from the metabolic intermediate acetol (Casazza *et al*., 1984; Phillips and Thornalley, 2005) (Fig. 6). The exogenous sources of MG include foods rich in carbohydrates like bread, potatoes and sucrose (Hayashi and Shibamoto, 1985). Cigarette smoking is other source of MG. Gas chromatographic analysis has shown levels of MG up to 59 µg/cigarette (Fujioka and Shibamoto, 2006).

1.3.b Reactivity of MG

MG is a highly reactive molecule. This is evident from a reported estimate that 99% of MG in the body is bound to proteins and other biomolecules whereas only about 1% is in a free form. The high reactivity of MG can be attributed to the electrophilic nature of the methyl group and to oxygen atoms (Fig. 1-5), which increase the positive charge on the carbonyl carbon of MG. Therefore, it becomes highly reactive in nucleophilic addition reactions than other dicarbonyl compounds (Shipar, 2006). Glyoxal and 3-deoxyglucosone (3-DG) are other reactive intermediate α-dicarbonyl compounds formed in the body. MG is an alkyl derivative of glyoxal and it has been reported that it is more reactive than glyoxal (Shipar, 2006). Due to its high reactivity MG can react with and modify certain proteins, DNA, lipids and other biomolecules and alter their structure and function. The binding characteristic of MG may also be different with different proteins (Dhar *et al*., 2009). Thus, an excess of MG and other dicarbonyl compounds in the body causes carbonyl overload and stress in conditions such as diabetes.

1.3.c Degradation of MG

Since MG is a highly reactive cytotoxic compound, the body has developed several detoxification mechanisms to degrade it. One of the major detoxification mechanisms is the highly efficient glyoxalase system. The glyoxalase system consists of two enzymes, glyoxalase I and glyoxalase II and catalytic amounts of reduced glutathione (GSH). The glyoxalase system is found in the cytosol of cells and cellular organelles, particularly mitochondria (Thornalley, 1990). MG reacts with GSH forming hemithioacetal. Hemithioacetal is converted to *S*-D-lactoyl-glutathione by glyoxalase I and then further metabolised into D-lactate by glyoxalse II and regenerating GSH (Thornalley, 1990).

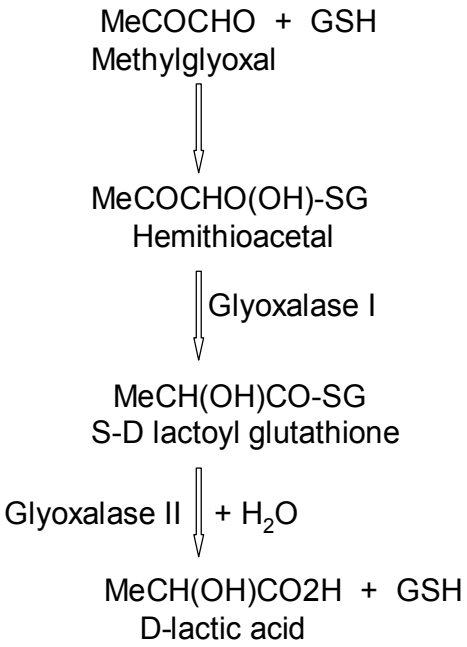

Figure 1-7. Pathway of MG metabolism [*Adapted from Biochem J 1990; 269(1): 1-11*]

Lactate is formed during anaerobic glycolysis using enzyme lactate dehydrogenase. Under conditions of decreased tissue oxygenation, lactic acid is produced as the anaerobic

cycle is utilized for energy production (Robergs *et al.*, 2004). Some studies have reported that cells over expressing glyoxalase I show enhanced tolerance to MG and exhibit better detoxification (Maclean *et al.*, 1998). Another family of enzymes called aldo-keto reductases (AKRs) reduce aldehydes and ketones. AKRs are a large superfamily of related proteins that carry out NADPH-dependent reduction of various aldehydes and ketones. The physiological function of most putative members of this superfamily is still unclear, partly due to their broad substrate specificity and partly because of the difficulty of genetic analysis, as many organisms have multiple genes that encode AKRs (Xu *et al.*, 2006).

Another minor pathway of MG detoxification is *via* MG dehydrogenase. MG dehydrogenase catalyzes the oxidation of MG to pyruvate using NAD or NADP as a cofactor (Ray and Ray, 1982).

1.3.d MG levels in pathological conditions

MG is formed as a byproduct during glycolysis. The plasma MG levels under normal conditions are between 0.5-1.5μM (Jia & Wu, 2007; Wang *et al.*, 2007). and the levels increase as much as 2 to 4 fold under diabetic conditions (Wang *et al.*, 2007; Beisswenger *et al.*, 1999). There is an increased body of evidence suggesting that increased MG formation in diabetes mellitus is linked to diabetic complications like retinopathy and nephropathy but the exact mechanism is far from clear. Previous studies have shown that MG levels are increased in plasma of SHR rats (13.8 μM) at 8 weeks of age and 33 μM at 20 weeks as compared to WKY at the same age (9.1 and 14.2μM). MG levels were also elevated in the aorta (2.5 nmol/mg protein) and kidney (0.3 nmol/mg protein) compared to WKY (1.5 nmol/mg protein) (0.2 nmol/mg protein) in an age dependent manner (Wang *et al.*, 2004; Wang *et al.*, 2005).

There was a significant increase in MG levels in serum, 3.2 µM as compared to 1.8 µM in control rats, and adipose tissue (4.2 µmol/g protein) of SD rats fed with high fructose diet for 9 weeks as compared to control rats (1.5 µmol/g protein) and the effect was attenuated by MG scavenger N-acetyl cysteine (NAC) (Jia and Wu, 2007). We have shown previously that incubation of vascular smooth muscle cells (VSMCs, A-10) with high glucose increases MG formation in a concentration and time dependent manner along with an increase in oxidative stress (Dhar *et al*., 2008). The western diet has increasing amounts of carbohydrates and the rapidly increasing incidence of childhood obesity and diabetes mellitus have become a major health concern (Van dam *et al*., 2002). In the absence of a genetic predisposition the link between high carbohydrate intake and the development of diabetes mellitus is unknown at a mechanistic level.

1.3.e Substrates involved in MG formation:

D-Glucose

Glucose is a monosaccharide, an important carbohydrate. All living cells use it as a main source of energy. There are two stereo-isomers of aldohexose which are D and L-glucose and D-glucose is biologically active. D-glucose is often referred to as dextrose monohydrate or dextrose. Under normal physiological conditions glucose levels are around 5mM but are elevated as much as 25mM under diabetic conditions (www.who.int/diabetes) (Tirosh *et al.*, 2005). During glycolytic process (breakdown of glucose), MG is formed as intermediate product. Elevated levels of MG have been reported in diabetic patients which correlate with the degree of hyperglycemia (Wang *et al*., 2008). MG levels are elevated during hyperglycemic (high glucose) conditions. Incubation of vascular smooth muscle cells with

high glucose for 3 h increases MG formation (Dhar *et al*., 2008). In human umbilical vein endothelial cells and rat aortic endothelial cells, incubation of 25mM glucose for 24 h increases MG formation and induces endothelial dysfunction (Dhar *et al*., 2010). Endothelial dysfunction is one of the features of diabetes mellitus. Previous studies have shown that incubation of human mononuclear cells (HMNCs) with MG and glucose induces apoptosis or necrosis. Apoptosis is triggered through multiple biochemical changes including reactive oxygen species (ROS) generation, activation of caspase 3 and DNA fragmentation (Hsieh and Chan, 2009). Increased glucose levels in diabetic patients have been shown to cause non-enzymatic glycation (Thomas *et al*., 2005).

Fructose

Fructose is also a monosaccharide and a simple reducing sugar. Fructose undergoes Maillard reaction (chemical reaction between amino acid and reducing sugars in the presence of heat). Because fructose exists to a greater extent in the open-chain form than does glucose, the initial stages of the Maillard reaction occurs more rapidly than with glucose. Therefore, fructose potentially may contribute to changes in food palatability, as well as other nutritional effects, such as excessive browning, volume and tenderness reduction during cake preparation, and formation of mutagenic compounds (Gaby, 2005). Feeding of normal SD-rats with fructose increased endogenous MG levels, impaired insulin signalling and reduced glucose uptake and the effect is mediated through the formation of MG as incubation of 3T3 adipocytes directly with MG impaired IRS/PI3K pathway, reduced glucose uptake and the effect was attenuated by pretreatment with MG scavenger N-acetyl cysteine (NAC) (Jia *et al*., 2006). Normal SD rats fed chronically with fructose for 16 weeks develop hypertension with

higher serum MG levels. The effects were attenuated by metformin possibly by scavenging MG (Wang *et al*., 2008). Incubation of vascular smooth muscle cells with 25mM fructose for 3 h increased MG formation significantly as compared to untreated control cells (Dhar *et al*., 2008). Increased fructose consumption as seen in Western diets may be a health concern. Because currently the main tendency is to focus on glucose in terms of increased MG, AGEs, and oxidative stress in conditions such as diabetes, it is important to consider the contribution of other substrates such as fructose, acetol, and aminoacetone to MG, AGEs, and oxidative stress load.

Aminoactone

Aminoacetone (AA) is the product of mitochondrial metabolism of threonine and glycine. AA is a threonine metabolite accumulated in threonemia, and diabetes. Oxidation of AA to MG, NH_4^+, and H_2O_2 has been reported to be catalyzed by a copper-dependent semicarbazide sensitive amine oxidase (SSAO) as well as by Cu(II) ions. H_2O_2 generated by the auto-oxidation of aminoacetone reacts with Cu(I) to form reactive species capable of causing oxidative DNA damage (Hiraku *et al*., 1999). SSAO catalyzes the conversion of aminoacetone to MG. SSAO is copper containing amine:oxygen reductase. SSAO has a wide distribution however is most abundant in vascular smooth muscle cells (Magyar *et al*., 2001). Previous studies have shown that AA can be deaminated to MG by amine oxidase in homogenates of various guinea pig tissues and in goat and ox plasma (Lyles and Taneja, 1987; Deng and Yu, 1999). We have reported previously increased formation of MG in VSMCs treated with different concentrations of AA. The amount of MG formed in VSMCs depends a great deal on the nature of the substrate. The important finding of our study is that

aminoacetone has the potential to form significant amounts of MG, carboxyethyl lysine (CEL), nitric oxide (NO), and oxidized DCF (peroxynitrite) even at low concentrations (Dhar *et al*., 2008). Because aminoacetone is an intermediate of protein metabolism, the production of MG and peroxynitrite under conditions of increased protein catabolism is worthy of further investigation.

Acetone

Acetone is an organic compound with a formula of OC $(CH_3)_2$. Acetone is a colorless flammable liquid and is a ketone. Small amounts of acetone are produced in the body by the decarboxylation of ketone. Once acetone is formed, it can be metabolized in several pathways. One of these pathways involves conversion of acetone to MG in two consecutive steps via acetol as intermediate. Acetol and MG are intermediates of the intrahepatic metabolism of acetone leading to pyruvate formation (Kalapos, 1999). The production of MG from acetone was proposed in 1984, based on studies of acetone metabolism in the rat (Casazza *et al*., 1984). The possible role of cytochrome P450s in acetone metabolism was recognized in 1980 (Coleman, 1980), and cytochrome P450 2E1 was identified as both the acetone and acetol monooxygenases in liver microsomes from rabbit, rat and mouse (Koop *et al*., 1985, Johansson *et al*., 1986). This pathway is inducible, and can be induced by several agents (acetone, ethanol, pyrazole, imidazole, etc.) or under different physiological and pathological circumstances, such as fasting or diabetes mellitus (Gonzalez and Lee, 1996).

1.3.f Methods of MG measurement

Number of methods have been reported for the measurement of MG in cultured cells,

plasma and tissue samples. Numerous studies have reported variable levels of MG in cultured cells, tissue and plasma samples of animals. These variabilities can be due to differences in protocol and sample preparation method. One of the most common and widely used method involves derivatization of MG with 1,2-diaminobenzene derivatives, such as *o*-PD, and the quantification of the resulting quinoxaline with high performance liquid chromatography (HPLC) (Chaplen *et al*., 1998). The high reactivity of MG makes it difficult for reproducible quantification from sample to sample. It is believed that less than 1% of MG exists in a free form while more than 99% exists in a protein-bound form (Ahmed *et al*.,, 2002; Chaplen *et al*., 1998). Previous studies have also proposed that the protein bound form can exist as an irreversibly bound pool or a reversibly bound pool. In this case the irreversibly-bound form remains stable under harsh assay conditions and therefore cannot be detected (Chaplen *et al*., 1998). Irreversibly-bound MG, characterized as AGEs, is detected by separate assays for AGEs (Ahmed *et al*., 2002; Nagaraj *et al*., 1996). The reversibly-bound MG appears to be in dynamic equilibrium with free MG and can be measured (Chaplen *et al*., 1998). However, the reversibly bound MG compounds are unstable and are, therefore, possibly a source of error in assays (Chaplen *et al*., 1998). Perchloric acid (PCA) is used in the protocol to stop metabolic reactions in the sample and to precipitate proteins which are immediately removed from the sample before it is derivatized with *o*-PD (Chaplen *et al*., 1996; Chaplen *et al*., 1998). Chaplen *et al* (1998) theorized that this will also precipitate the reversibly-bound MG adducts and remove them from the sample along with the proteins that are removed after centrifugation. According to the results obtained by Chaplen *et al* (1998), a longer incubation of the precipitated proteins, which will include the reversible MG adducts, will allow the acidic environment to free MG from its reversible binding and make it amenable to detection.

Variations in the sample treatment protocol detected up to an amazing 100 to 1000 fold more MG from the same sample, when applied to cultured Chinese hamster ovary (CHO) cells (Chaplen *et al*., 1998). However, it is not known if similar variations in protocol would affect the amount of MG detected in other sample types such as plasma, body organs or tissues and other cultured cells such as vascular smooth muscle cells (VSMCs).

1.3.g Range of doses and concentrations of MG used experimentally

The physiological concentration of MG in normal rats is between 0.5-10 μM (Wang *et al*., 2005; Wang *et al*., 2004). Since MG is formed during glycolysis, the intracellular levels of MG are higher than circulating levels. Numbers of studies have used higher concentrations and doses of MG. For example, a 1mM concentration of MG in rat pancreatic β cells caused rapid depolarization, elevated intracellular Ca^{2+} concentration and acidification in intact islets (Cook *et al*., 1998). MG concentration of up to 500 μM has been used to study MG induced apoptosis of jurkat cells. Sheader *et al* (2001) used a concentration between 0.1-10mM to study the effect of MG on insulin secreting cells (Sheader *et al*., 2001). In one *in vivo* study by Jerzykowski *et al*., a dose of up to 500 mg/kg MG has been used to see its effect on plasma glucose levels in cats (Jerzykowski *et al*., 1975). MG concentration between 2.5-5mM have been used to study the effect of MG on insulin signalling pathway in L6 muscle cells *in vitro* in cell culture studies (Chavey *et al*., 2006). Similar high concentrations of exogenous MG have been employed in most *in vivo* and *in vitro* studies, which were not grossly aimed at studying the toxic effects of MG (Cook *et al*., 1998). This raises an important concern of whether these studies bear physiological or pathological relevance. However in normal healthy people, MG is not a concern because the highly efficient glyoxalase system degrades

it and coverts it into D-lactate (Desai & Wu, 2007).

1.3.h Advanced glycation endproducts

MG, 3-deoxy glucosone and glyoxal are major precursors of advanced glycation endproducts (AGEs). AGEs are formed when MG reacts with certain proteins and alters their structure and function. AGEs play a significant role in causing the pathologic changes in many conditions like aging and diabetic complications such as micro vascular complications, retinopathy, neuropathy and nephropathy (Desai and Wu, 2007). AGE formation is a non-enzymatic glycation reaction between reducing sugars and amino groups of proteins. These phenomena lead to browning, fluorescence and cross-linking of proteins (Desai & Wu, 2007). The glycation reaction is initiated with the reversible formation of a Schiff base which undergoes further rearrangement to form a stable Amadori product (Desai & Wu, 2007). The Amadori product undergoes further reactions with dicarbonyl intermediates to form AGEs (Tomoko *et al*., 1999). Since the product formed is brown in colour, it is also known as "browning reaction" (Maillard, 1916). MG is one of the intermediates in Maillard reaction both i*n vitro* and *in vivo*.

A number of AGEs are formed under various *in vitro* or *in vivo* conditions. Some of the most common non-cross-linking AGEs are *N*- carboxymethyl-lysine (CML) (Ahmed *et al*., 1986; Reddy *et al*., 1995), carboxymethylvaline (CMV) (Cai and Hurst, 1999), *N*-carboxyethyl-lysine (CEL) (Ahmed *et al*., 1997) and pyralline (Miyata and Monnier, 1992). The fluorescent cross-linking AGEs are pentosidine (Sell and Monnier, 1989) and crossline (Obayashi *et al*., 1996) and non-fluorescent cross-linking AGEs are argpyrimidine (Shipanova *et al*., 1997) (Glomb *et al*., 2001), methylglyoxal-lysine dimer (Chellan and Nagaraj, 1999)

and imidazolones (Konishi *et al*., 1994). One of the non specific AGEs found routinely in disease conditions is CML and these can be detected immuno-histochemically in tissues and cultured cells.

AGEs are markers of oxidative stress. A number of AGE inhibitors or AGE breakers have been developed over the years and they act by targeting multiple steps of AGE formation. Some of the examples of AGE inhibitors are aminoguanidine, N-acetyl cysteine (NAC) and aspirin. AGE breakers act by breaking α-dicarbonyl crosslinks. One of the most promising AGE breaking compounds developed by Alteon is ALT-711 (Alagebrium) (Desai and Wu, 2007).

1.3.i MG and insulin signalling

Insulin, a peptide hormone plays a major role in regulating pathways leading to cellular proliferation and differentiation. When insulin binds to its receptor (IR), the receptor tyrosine kinase is activated, leading to phosphorylation of several intracellular proteins. These rapid events generate multiple signalling cascades that eventuate in the final cellular responses to insulin. Insulin activated signalling molecules are Ras/Raf, PI3K/AKT and PKC pathway (Taniguchi *et al*., 2006; Kruger *et al*., 2008). Insulin resistance is a common abnormality in disease situations like diabetes and obesity. Chronic hyperglycemia leading to insulin resistance is the primary etiological factor in the pathogenesis of diabetic microvascular and macrovascular complications (Vuorinen-Markkola *et al*., 1992). One of the mechanisms of hyperglycemia induced damage in diabetes is AGEs. Large number of substantial data indicates that glucose toxicity may be the consequence of increased formation of MG. Increased formation of MG may occur due to increased availability of its precursors like

increased glucose during diabetes, increased acetone and acetol during diabetic ketoacidosis and more of threonine in gout and hyperuricemia. Exposure of cultured L6 muscle cells with MG induces inhibition of insulin stimulated phosphorylation of PKB and ERK1/2 (Chavey *et al*., 2006**).** MG modifies the B-chain of human insulin *in vitro*, and that modification occurs predominantly at the N terminus and arginine residue via Schiff base formation. The extent of modification increases with the relative concentration of MG. The formation of MG-insulin adducts leads to the reduction of insulin-mediated glucose uptake by its target cells or tissues, impairs autocrine control of insulin release from pancreatic ß-cells, and decreased hepatic clearance of insulin from liver cells (Jia *et al*., 2006). Increased formation of MG has been observed *in vivo* in serum and adipose tissue of fructose fed rats. A significant reduction in IRS-1 tyrosine phosphorylation and PI3K activity was observed in cultured 3T3 adipocytes treated with MG. The effects of MG were reversed by N-acetyl cysteine (NAC), an MG scavenger, which supports the role of MG in the impaired insulin stimulated glucose uptake (Jia and Wu, 2007). Moreover, MG levels in the body are dependent on the balance between its synthesis and degradation. When the formation of MG exceeds its degradation, accumulation of MG occurs even with normal concentration of its precursors. Also accumulation of MG occurs when the degradative capacity of the glyoxalase system is compromised. Increased MG formation has been observed after application of glyoxalase-I inhibitors (Creighton *et al*., 2003). Clarification of the role of MG in the development of insulin resistance may lead to a discovery of new mechanisms and methods for the management and prevention of insulin-resistance syndrome, including diabetes and hypertension.

1.3.j MG and Oxidative stress

Numerous *in vitro* and *in vivo* studies have reported MG induces oxidative stress. The markers used to measure oxidative stress are superoxide, hydrogen peroxide and peroxynitrite levels (Desai and Wu, 2008). Increased superoxide production has been observed in aorta of SHR which was associated with elevated plasma MG as compared to age matched WKY rats (Wang *et al*., 2005). Incubation of VSMCs with exogenous MG induces superoxide formation in a concentration and time dependent manner (Chang *et al*., 2005). These effects of MG were reversed by superoxide scavenger SOD or NADPH-oxidase inhibitor diphenylene iodonium (DPI) (Wang *et al*., 2006). Previous studies have shown that incubation of VSMCs with MG increased the generation of H_2O_2. The effects of MG were prevented by NAC and GSH, both act as scavengers of MG. In 4-13 week old SHR rats, increased blood pressure was associated with increased NO production, increased iNOS expression and reduced eNOS expression in aorta along with elevated aortic and plasma MG levels as compared to age matched WKY rats (Wang *et al*., 2006). Increased Reactive Oxygen Species (ROS) generation has been observed in various insulin resistant and cardiovascular disease conditions. Excess superoxide (O_2•-) can react with NO to form peroxynitrite ($ONOO^-$) (Fig. 8). Peroxynitrite is a reactive oxidising and nitrating agent and it can damage wide range of molecules including DNA and proteins (Pacher *et al.*, 2007).

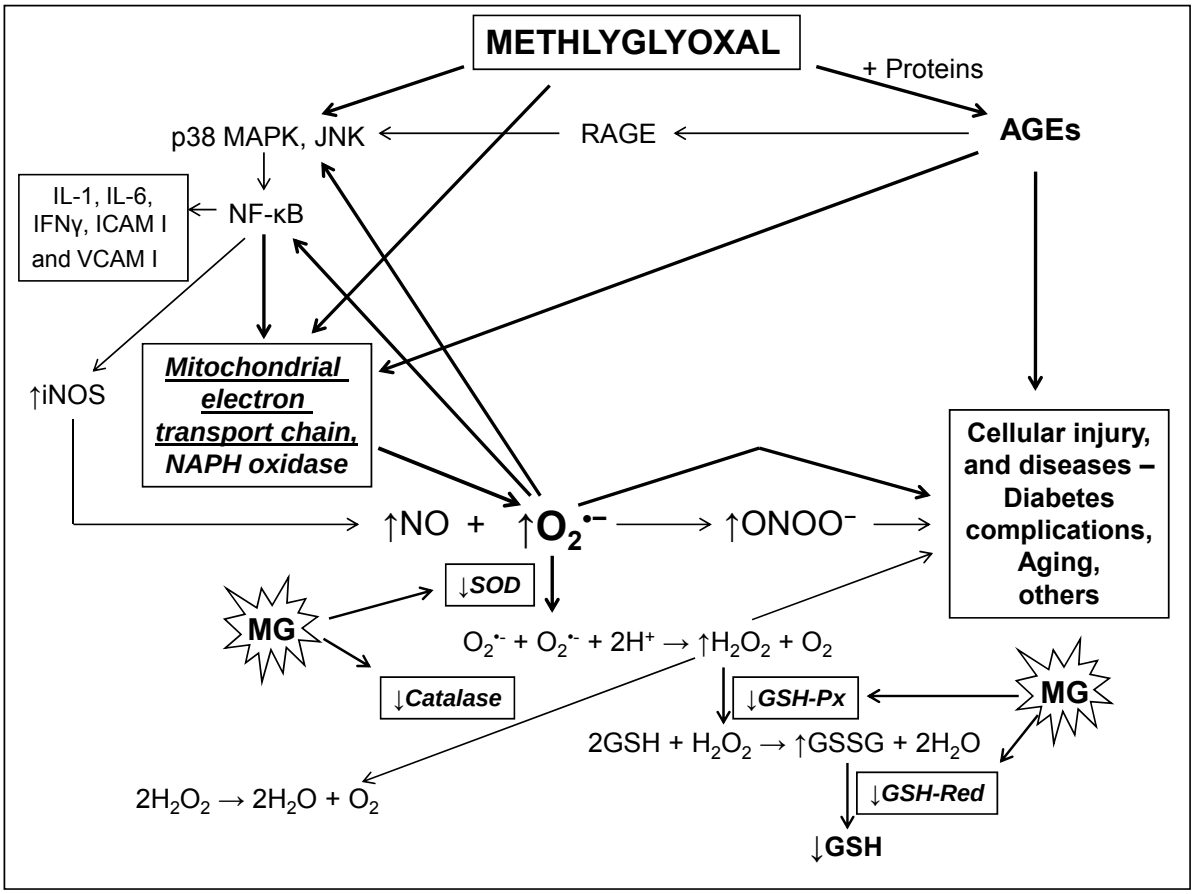

Figure 1-8: Schematic showing mechanisms of MG induced oxidative stress.

MG induces oxidative stress through multiple mechanisms and pathways as shown in the figure. Free radicals and reactive oxygen species such as superoxide, peroxynitrite and hydrogen peroxide, and advanced glycation endproducts (AGEs) cause cellular injury, pathological conditions and diseases.

Abbreviations: AGEs, advanced glycation end products; GSH, reduced glutathione; GSH-Px, glutathione peroxidase; GSH-Red, glutathione reductase; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; ICAM 1, intracellular adhesion molecule 1; IFNγ, interferon γ; IL1, interleukin 1; JNK, JUN N-terminal kinase; MG, methylglyoxal; NF-κB, nuclear factor-kappaB; NO, nitric oxide; $O_2^{\bullet-}$, superoxide anion; $ONOO^-$, peroxynitrite; p38 MAPK, p38 mitogen activated protein kinase; RAGE, receptor for advanced glycation endproduct; SOD,

superoxide dismutase; VCAM 1, vascular cell adhesion molecule 1.

[***Adapted from Drug Metabol Drug Interact. 2008;23(1-2):151-73***.]

MG also induces oxidative stress by inducing cytokine IL-1 beta in cultured rat hippocampal neuronal cells. The activity of antioxidant enzymes superoxide dismutase (SOD), catalase and glyoxalase has been shown to be affected by MG *in vitro*. MG is mainly detoxified by glyoxalase system which requires catalytic amount of reduced glutathione for its activity. High glucose and oxidative stress have been shown to reduce GSH levels (Kikuchi *et al*., 1999). MG has also been shown to reduce the activity of glutathione peroxidase and glutathione reductase (Blakytny and Harding, 1992; Paget, 1998) which in turn recycle the oxidized glutathione (GSSG) back to GSH. This phenomenon impairs the detoxification of MG, increases its half-life and adds to further oxidant damage. Morphological changes have been observed in human umbilical vein endothelial cells (HUVECs) with MG in a concentration dependent manner (Chan and Wu, 2008).

High concentrations of MG upto 5mM induce apoptosis in bovine aortic endothelial cells (BAECs) by impairing mitochondrial membrane potential, elevation of caspase-3, thus inducing oxidative stress and ultimately apoptosis (Takahashi *et al*., 2010). MG levels are high in the cerebrospinal fluid (CSF) of neurodegenerative patients and hippocampus is found to be vulnerable to oxidative damage. MG treatment caused an inhibition of antioxidant defence mechanism and detoxification processes, shifting the cellular environment towards a more oxidized state and causing the damage (Desai *et al*., 2010). However most of the studies done so far have studied *in vitro* effects of MG in cultured cells. Whether MG directly induces similar changes *in vivo* are not known.

1.3.k Agents that inhibit MG formation

A number of agents have been tested in the past for scavenging MG directly or by inhibiting AGE formation. Here we will discuss some of the agents which have been used in the past and which might be used in the near future.

Aminoguanidine (AG)

Aminoguanidine is the best known and most widely used AGEs inhibitor. Aminoguanidine was first known to prevent diabetes induced arterial wall protein cross linking. Aminoguanidine inhibits the 'cross-linking' (or glycosylation) of proteins. It acts by inhibiting the formation of amadori products by reacting with dicarbonyl compounds like MG (Thornalley, 2003). Aminoguanidine is an irreversible inhibitor of SSAO, an enzyme which catalyses the conversion of aminoacetone to MG (Casazza *et al*., 1984). Aminoguanidine also reduces oxidative stress by inhibiting peroxynitrite and inducible nitric oxide synthase formation (Szabo *et al*., 1997) (Misko *et al*., 1993). Aminoguanidine prevents AGEs formation *in vitro* as well as *in vivo*. The doses used *in vivo* range from 25 mg/kg/day to 50 mg/kg/day and up to 100 mg/kg/day. Aminoguanidine has also been shown to prevent oxidative modification of low density lipoproteins by binding reactive aldehydes formed during lipid peroxidation and preventing their subsequent conjugation to apo B. Previous studies have also shown that aminoaguanidine treatment decreases age related cardiovascular damage in rats. There was a decrease AGE formation in cardiac, renal and aortic tissue of rats treated with aminoguanidine. Age related cardiac hypertrophy and aortic stiffening was also prevented by aminoaguanidine administration in comparison to untreated rats (Li *et al*., 1996). Recent studies have also shown that aminoaguanidine treatment reduces the age dependent

development of hypertension in spontaneously hypertensive (SHR) rats by scavenging MG (Wang *et al*., 2007). However further studies are required to investigate the causative role of MG and MG related AGEs in hypertension.

Metformin

Metformin is an oral antihyperglycemic agent for the treatment of type II diabetes. It is a biguanide with structure similar to aminoguanidine. In a study done in type II diabetic patients, metformin lowered MG levels in these patients (Tanaka *et al*., 1999). Metformin is also shown to be a potent inhibitor of glycation. Chronic treatment with metformin reduces AGE formation in lens, kidney and nerves in diabetic rats. It has also been reported that metformin reduces MG formation in dose dependent manner (Beisswenger *et al*., 1999). There are several mechanisms by which metformin inhibit glycation processes. One mechanism is that metformin acts by binding its guanidine group to the α-dicarbonyl group of MG. In one *in vitro* study metformin directly reacts with MG to form stable triazepinone derivatives (Lopez *et al*., 1999). Another study has shown that metformin reduces MG levels by increasing MG detoxification via increased glyoxalase pathway (Rahbar *et al*., 2000).

Alagebrium (ALT-711)

Alagebrium (4,5-dimethylthiazolium, ALA) (formerly known as ALT-711) (Fig. 1) is a novel advanced glycation endproducts (AGEs) cross-link breaking compound which has been studied mainly for its chronic effects on AGEs (Coughlan *et al*., 2007; Guo *et al*., 2009; Little *et al*., 2005; Peppa *et al*., 2006; Susic *et al*., 2004; Thallas-Bonke *et al*., 2004; Wolffenbuttel *et al*., 1998; Ulrich and Zhang, 1997; Zieman *et al*., 2007). The first AGEs cross-link breaking

compound discovered was phenacylthiazolium bromide (PTB) in 1996. PTB reacts with and cleaves covalent cross-links of AGEs-derived proteins. PTB degrades rapidly and hence a more stable derivative alagebrium was developed. ALA (210 mg/kg twice a day for 8 weeks) given to patients with systolic hypertension reduced vascular fibrosis and markers of inflammation (Zieman *et al*., 2007). Intraperitoneal injection of ALA (1 mg/kg) daily for 1 or 3 weeks reversed diabetes-induced increase of arterial stiffness measured by *in vivo* and *in vitro* parameters in STZ-induced diabetic rats, and improved impaired cardiovascular function in older rhesus monkeys (Wolffenbuttel *et al*., 1998; Ulrich, 1997). ALA (10 mg/kg for 16 weeks) also increased glutathione peroxidase and superoxide dismutase activities in aging rats and reduced oxidative stress (Guo *et al*., 2009). The Alteon Corporation in USA is developing alagebrium (ALT-711, thiazolium salt) as an antiaging drug. ALT-711 is currently being tested in human clinical trials. However, it has not been shown yet if ALA has acute effects against precursors of AGEs such as methylglyoxal (MG) and glyoxal.

N-acetyl cysteine

NAC is another agent that can scavenge MG (Jia and Wu, 2007; Vasdev *et al*., 1998). NAC a cysteine containing thiol compound and MG binds with high affinity to cysteine. NAC has other beneficial properties, e.g. it can increase GSH levels, which itself is the prime MG scavenger in the body (McLellan *et al*., 1994; Desai and Wu, 2007), and it can also directly react with free radicals and thus acts as an antioxidant (Aruoma *et al*., 1989; Dekhuijzen, 2004). NAC is already used clinically for other conditions such as acetaminophen overdose (Millea, 2009).

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

CHAPTER 2

2.1 Hypothesis

Rationale for hypothesis

Studies have reported elevated levels of MG in hyperglycemic and hypertensive rats (Wang *et al*., 2005; Jia and Wu, 2007). The plasma levels of MG have been reported to be increased 2-4 fold in diabetic patients (Wang *et al*., 2007a; McLellan *et al*., 1994). *In vitro* studies have shown that MG impairs the functioning of the insulin molecule and second messengers of the insulin signalling pathway in cultured cells (Jia *et al*., 2006, Jia and Wu, 2007; Ribolet-Chavey *et al*., 2006). However, elevated plasma levels in diabetic patients and fructose-fed rats are associative findings and do not establish a cause-effect relationship. Moreover, the effects of MG, especially high concentrations of MG employed, on insulin signalling in cultured cells cannot be extrapolated to *in vivo* situations. *In vivo* studies on the effects of exogenously administered MG to animals have not been performed to evaluate its role in the pathogenesis of insulin resistance. Thus, it is not known if the elevated MG levels in diabetic patients are the cause of diabetes or an effect of diabetes or both?

Based on the above rationale the following hypothesis was formed:

<u>Hypothesis</u>: A pathological elevation of methylglyoxal in the body is one of the causative factors in the development of insulin resistance, endothelial dysfunction and type 2 diabetes mellitus.

I designed protocols to perform a comprehensive study on the effects of exogenously administered MG to normal male Sprague-Dawley rats, on glucose tolerance and adipose

tissue glucose uptake, and pancreatic islet insulin secretory machinery.

My project evolved by following the objectives and experimental approaches described below:

2.2 Objectives and experimental approach

2.2.a Comparison of methylglyoxal production in vascular smooth muscle cells from different metabolic precursors

Although MG production from glucose has been well documented, the relative contribution of other intermediates of different metabolic pathways to MG formation is far less known. My aim was to determine and compare the formation of MG, MG-induced AGE, N^{ε}-carboxyethyl-lysine (CEL), inducible nitric oxide synthase (iNOS), nitric oxide and peroxynitrite from different metabolic precursors in cultured rat aortic VSMCs. The experimental approach was to incubate cultured A10 VSMCs with different concentrations of the known substrate d-glucose (Thornalley, 1996; Desai and Wu, 2007) for different times to determine the optimum concentration and incubation time. This concentration and incubation time was used for incubation of A10 cells with other putative substrates (Figure 1-6) to determine their relative potencies in generating MG and oxidative stress compared to d-glucose.

2.2.b Optimization of sample preparation protocol for the measurement of MG by HPLC

Variable plasma MG values have been reported in the literature. In the body MG is free, reversibly bound or irreversibly bound with cellular constituents (mostly with proteins)

(Mugo and Bottaro, 2008; Chaplen *et al*., 1998). The most widely used method, *viz*. high performance liquid chromatography (HPLC), detects the free MG levels in a given sample (Chaplen *et al*., 1998). It has been proposed that incubation of the sample with an acid frees up the reversibly bound MG, which is detected, and that the longer the incubation time with the acid, the greater is the amount of reversibly-bound MG released (Chaplen *et al*., 1998). To test this proposal, several different protocols were tested on different biological samples. The aim was to define a protocol/s that provided reproducible and consistent values of MG in a biological sample.

2.2.c To investigate whether exogenously administered MG has acute effects on glucose homeostasis in Sprague-Dawley rats, and whether alagebrium, an AGE-breaker, can prevent these acute effects of MG

MG was administered by three routes: oral gavage, intraperitoneal (i.p.) injection and intravenous (i.v.) injection to determine a dose and route that would give plasma MG levels within the range of 1-10 μM reported in the literature (Jia and Wu, 2007; Wang *et al*., 2008). Subsequently, a single dose of MG was administered to 12 week old male SD rats and after 2 h a glucose tolerance test (GTT) was performed to evaluate glucose tolerance. The adipose tissue and pancreatic islets were isolated to determine glucose uptake and insulin release, respectively. Alagebrium (ALA, formerly known as ALT-711) has been developed as an AGEs-breaker (Coughlan *et al*., 2007; Guo, Y *et al*., 2009; Wolffenbuttel *et al*., 1998). The acute effects of ALA on major precursors of AGEs such as methylglyoxal (MG) have not been reported. The preventive effects of ALA were examined by preadministration of ALA followed by MG in groups of rats. Distribution of exogenously administered MG to different

organs/tissues was measured.

2.2.d To investigate whether high glucose induced endothelial dysfunction is mediated by methylglyoxal

Hyperglycemia induces endothelial dysfunction by increasing oxidative stress (Potenza *et al.*, 2009, Triggle, 2008, Nishikawa *et al.*, 2000). The aim was to investigate whether MG induces endothelial dysfunction, and compare it with high glucose (25 mM)-induced endothelial dysfunction. Two models were used: isolated rat aortic rings from 12 week old male SD rats, and cultured endothelial cells from two species, viz. rat aorta and human umbilical vein. The parameters chosen as indicators of endothelial dysfunction were endothelium-dependent relaxation of isolated aortic rings, eNOS activity, protein expression, NO production, cyclic guanosine monophosphate (cGMP) levels, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and superoxide production in cultured rat aortic endothelial cells (RAECs) and human umbilical vein endothelial cells (HUVECs). Two types of compounds were used in an attempt to differentiate the direct effects of MG on eNOS and the indirect effects of oxidative stress induced by MG and high glucose on eNOS. These compounds were aminoguanidine, an MG scavenger, apocynin, an NADPH oxidase inhibitor and antioxidant, and NAC, which is an MG scavenger as well as an antioxidant.

2.2.e To investigate whether MG is a causative factor in the development of insulin resistance *in vivo* in SD rats

The chronic effects of MG on glucose homeostasis and pancreatic insulin secretory machinery were studied in 12 week old male SD rats. A comprehensive protocol was

developed that incorporated *in vivo*, *in vitro* and molecular techniques. GSH plays a vital role in the degradation of MG and keeps MG levels in the body within a physiological range. In one group of rats buthionine l-sulfoximine (BSO), a glutathione synthesis inhibitor, was co-administered with MG with a view to examine whether higher MG levels were achieved and whether the effects of administered MG, under study were compounded in this group. ALA co-treatment with MG was used to try and prevent any observed effects of MG. One novel feature of this study was administration of MG by continuous infusion *via* an osmotic minipump implanted subcutaneously on the back (Wang *et al*., 2006b). The purpose was to mimic the supposedly continuous generation of MG in the body and avoid the excessive plasma peaks of MG that inevitable arise from i.p. or i.v. administration.

CHAPTER 3

GENERAL METHODOLOGY

Cell culture

Rat thoracic aortic smooth muscle cell line (A-10 cells) was obtained from American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin at 37° C in a humidified atmosphere of 95% air and 5% CO_2, as described previously (Chang *et al.*, 2005). A-10 cells were seeded either in 100 mm dishes for MG measurement or in 96-well plates for other assays, with an equal amount of cells (10^6/ml) in each well, and cultured to confluence. For immunocytochemistry staining, cells were seeded on cover glass slides (2×10^6/ml). Cells were starved in FBS-free DMEM for 24 h before exposure to different metabolic precursors.

Rat aortic endothelial cells (RAECs) were isolated from male SD rats according to the method of McGuire, *et al* (Mcguire *et al.*, 1987). The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 0.15 mg/mL endothelial cell growth supplement (Biomedical Technologies Inc., MA, USA) at 37° C in a 95% air / 5% CO_2 incubator. For the initial culture matrigel (Sigma-Aldrich, Oakville, ON, Canada) coated 100-mm culture dishes were used. RAECs were identified by their typical cobblestone morphology and positive staining for von Willebrand factor. Cells between passage 3 and 6 were used for the experiments. Human umbilical vein endothelial cell (HUVEC) line was obtained from American Type Culture Collection and cultured in Kaighns F12K medium containing 10% fetal bovine serum (FBS), 0.1 mg/mL heparin and 0.03-0.05 mg/mL endothelial cell growth supplement at 37°C in a humidified atmosphere of 95% air and 5% CO_2. HUVECs were seeded either in 100-mm dishes or in 96-well plates for different assays, with an equal amount of cells (10^6/mL) in each well, and cultured to confluence.

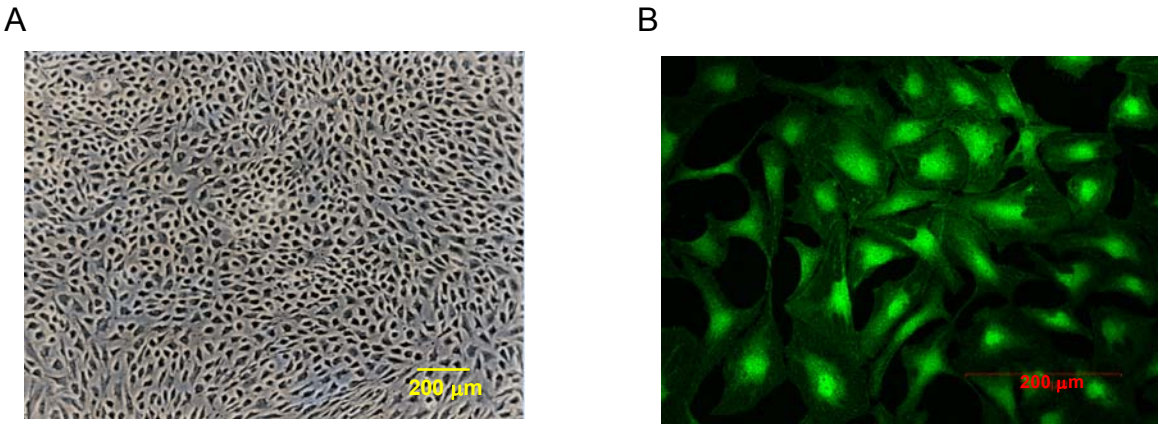

Fig. 1-9 Rat aortic endothelial cells were freshly isolated and cultured. The identity of endothelial cells was confirmed at the beginning of the culture by (A)) the typical cobblestone morphology of confluent cells and also by (B) positive staining for von Willebrand factor.

Animals

Male 11-week old Sprague-Dawley (SD) rats from Charles River Laboratories (Quebec, Canada) were used according to guidelines of the Canadian Council on Animal Care. After one week of acclimatization the rats were fasted overnight before the experiments. Carotid artery and jugular vein cannulation was done under halothane anaesthesia for blood collection and drug injection. At the end of each experiment, rats were euthanized with thiopental sodium (100mg/kg, i.p.). Blood samples were collected in EDTA containing tubes. Heart, liver, kidney, aorta, brain, spleen, adipose tissue and skeletal muscle were collected and snap frozen in liquid nitrogen and stored at -80 c for further processing.

MG measurement

MG was measured by a specific and sensitive high-performance liquid chromatography (HPLC) method (Dhar *et al*., 2009). MG was derivatized with *o*-phenylenediamine (*o*-PD) to form the quinoxaline product, 2-methylquinoxaline, which is very specific for MG (Chaplan *et al*., 1998). After the specified incubation time of the cultured cells with MG, the culture medium was aspirated completely and the cells were washed twice with phosphate buffered saline (PBS). The cells were scraped, centrifuged and cell pellets were resuspended in ice-cold PBS, and lysed over ice by sonication (5 s, three times). The sample was then incubated on ice for 24 h with 1 N perchloric acid (PCA) and 10mM *o*-PD. The sample was centrifuged (12,000 rpm, 15 min) to remove the PCA-precipitated material. The 2-methylquinoxaline and underived 5-methylquinoxaline, which was added to the samples as the internal standard, were quantified on a Hitachi D-7000 HPLC system (Hitachi, Ltd., Mississauga, ON, Canada) via Nova-Pak® C18 column (3.9×150 mm, and 4 µm particle diameter, MA, USA).

Measurement of peroxynitrite

The formation of peroxynitrite was determined by a sensitive dicholorofluorescin (DCFH) assay. Briefly, cells were loaded with a membrane-permeable, nonfluorescent probe 2',7'-dichlorofluorescin diacetate (CM-H2DCFDA, 5 µM) for 2 h at 37°C in FBS-free DMEM in the dark. After washing with PBS 3 times, cells were treated with or without different substrates or MG for different incubation times, and finally subjected to detection. Once inside the cells, CM-H2DCFDA becomes membrane-impermeable DCFH2 in the presence of cytosolic esterases, and is further oxidized by peroxynitrite to form the fluorescent

oxidized dichlorofluorescein (DCF). The probe has high reactivity with peroxynitrite and its products $CO_3^{\cdot -}$ and $NO_2^{\cdot}$ but is not entirely specific for it. It also has low reactivity for hydrogen peroxide and even lower for superoxide (Wardman, 2007).The fluorescence intensity was measured with excitation at 485 nm and emission at 527 nm utilizing a Fluoroskan Ascent plate reader (Thermo Labsystem) and Ascent software, and expressed in arbitrary units.

Measurement of nitrite and nitrate

Cells were incubated with different substrates or MG and then washed with PBS. The supernatant was used for the measurement of nitrite and nitrate with a fluorimetric assay kit (Calbiochem) based on the Greiss reaction. The assay is based on the enzymatic conversion of nitrate to nitrite by nitrate reductase followed by the addition of 2,3-diaminonaphthalene, which converts nitrite to a fluorescent compound. Fluorescence intensity measurements of this compound accurately determine the nitrite (NO_2) concentration (excitation max.: 365 nm; emission max.: 450 nm) (Nussler *et al*., 2006).

Immunocytochemistry /Immunohistochemistry

A-10 cells were seeded on glass cover slips followed by incubation with different substrates for 3 h, and subjected to iNOS and CEL staining. As described previously (Wang *et al*., 2007), the treated cells were fixed in 4% paraformaldehyde for 30 min at room temperature and washed twice with 0.01 N phosphate buffered saline (PBS). After permeation with 0.1% Triton X-100 for 5 min and two washes with PBS, the cells were incubated with normal goat serum (diluted 1:30 in 0.1 N PBS) for 1 h to block non-specific binding sites.

After shaking off the goat serum the slides were incubated with iNOS antibody (1:500; BD Transduction Laboratories) and CEL antibody (1:100; a generous gift from Novo Nordisk, A/S, Denmark) overnight at room temperature. Cells were washed twice in PBS (0.01 N) for 5 min and incubated with secondary fluorescein isothiocynate (FITC) conjugated anti-CEL and Texas red conjugated anti-iNOS antibodies (Molecular Probes) for 2 h. After washing thrice with PBS the slides were mounted in glycerol:PBS (3:7), coverslipped and observed under a fluorescence microscope. Staining intensity was quantified using the Metamorph image analysis software (*v*. 7, Molecular Devices). Slides from four different experiments were analyzed with 5 fields per slide observed and averaged.

Intravenous Glucose Tolerance Test (IVGTT)

After overnight fasting, an intravenous glucose tolerance test (IVGTT) was performed as described previously (Laight *et al*., 1999). Briefly, the trachea, left jugular vein, and right carotid artery were cannulated in anesthetised rats. After collecting a basal blood sample rats were treated with saline, MG or MG+ALA. After 2 h a 0 min blood sample was taken and a bolus dose of glucose (0.5 g/kg) was given i.v. and further blood samples were collected at 1, 3, 6, 12 and 24 min from the carotid artery. Plasma glucose levels were determined using a glucose assay kit (BioAssay Systems, Hayward, CA, USA) and insulin levels were measured with a rat insulin assay kit (Mercodia Rat Insulin ELISA). The IVGTT result was calculated as the area under the curve (AUC) for both plasma glucose and insulin levels between time 0 min and 24 min and expressed as arbitrary units.

Glucose uptake

Insulin sensitivity of adipose tissue was evaluated by measuring insulin-induced 2-Deoxy-[^{3}H] glucose (2-DOG) uptake as described previously (Jia *et al*., 2006). Briefly, abdominal visceral adipose tissue was chopped and digested in DMEM base (no glucose, no serum) with collagenase (1.5mg/ml) at 37° for 20 min. The mixture was filtered, centrifuged, supernatant discarded and the pellet was re-suspended in the same DMEM. Thereafter, the cells were exposed to 100 nM insulin for 30 min and continuously incubated for another 20 min after the addition of [^{3}H]-2-DOG (0.1 µCi/500µl) with glucose (50 µM) to the medium. The incubation was stopped by washing cells three times with ice-cold glucose-free phosphate buffer. The cells were lysed in 0.1% sodium dodecyl sulphate (SDS) and 1 N NaOH and transferred into scintillation vials for counting (Beckman LS 3801 scintillation counter).

Preparation of total membrane fraction from adipose tissue for GLUT4

Abdominal visceral adipose tissue isolated from rats was homogenized in buffer B [10 mmol/l Tris–HCl, 1 mmol/l EDTA, 250 mmol/l sucrose and 0.1 mmol/l phenylmethylsulfonyl fluoride (PMSF, pH 7.4)] using a polytron homogenizer. The homogenate was centrifuged at 1700×*g* for 10 min at 4° C and the resulting supernatant was centrifuged at 8600 x *g* for 10 min at 4° C. The supernatant was then centrifuged at 185000 x *g* for 60 min at 4 °C, and stored at −70° C before use. The protein concentration of the supernatant was determined by the bicinchoninic acid (BCA) protein assay reagent.

Immunoprecipitation and western blotting

For immunoprecipitation abdominal visceral adipose tissue was lysed in an ice-cold

radioimmunoprecipitation assay buffer (RIPA) buffer containing 30 mM HEPES (pH 7.4), 5 mM EDTA; 1% Nonidet P-40, 1% Triton X-100, 0.5% sodium deoxycholate, 8 mM Na_3VO_4, 1 mM NaF and 2 mM protease inhibitor (Jia and Wu, 2007). Tissue homogenates were incubated with IRS-1 antibody for two hours at 4º C, followed by incubation with Protein A/G-Agarose for further two hours at 4º C. Immunoprecipitates were separated using spin-collection filters and washed once with RIPA buffer and three times with PBS. For western blotting, cell lysates or membrane fractions (50 µg) were boiled with sample buffer for 5 min, resolved by 10–12% SDS-PAGE, and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). The membranes were blocked and incubated with the anti-IR (1:500) (Santa Cruz, CA, USA), anti-GLUT4 (1:500) (Santa Cruz, CA, USA), and anti-*β*-actin antibodies (1:2000) (Santa Cruz, CA, USA), respectively, followed by incubation with horse radish peroxidase conjugated secondary antibodies (1:3000) (Upstate, MA, USA). The proteins were then visualized with chemiluminescence reagents (Amersham Biosciences, NJ, USA) and exposed to X-ray film (Kodak Scientific Imaging film, X-Omat Blue XB-1).

Isometric tension studies on aortic rings

Isometric tension studies were carried out on rat aortic rings as described (Wu *et al*., 1998). Briefly, the thoracic aorta was rapidly removed from different groups of SD rats and placed in Krebs solution. The aorta was cleaned gently off extra tissue and cut into rings 3-4 mm in length and mounted in 10 mL organ baths containing Krebs solution maintained at 37° C and bubbled with 95% O_2 + 5% CO_2. Aortic rings were equilibrated for 90 min at a resting tension of 2 g, the bathing solution being renewed every 15 min. To obtain relaxant responses the rings were pre-contracted with phenylephrine (1 µM) and concentration-dependent

relaxation in response to acetylcholine (ACh) was obtained. Sets of rings were incubated with Krebs alone (control, 5 mM glucose) or Krebs containing glucose (15 or 25 mM) or MG (30 or 100 μM) for 2 h before obtaining dose-related responses with ACh. One set of rings was co-incubated with the MG scavenger aminoguanidine (AG, 100 μM) for 2 h. Isometric tension was measured with isometric force transducers with the 'Chart' software and Powerlab equipment (AD Instruments Inc., Colorado Springs, CO, USA).

eNOS activity assay

The NOS activity assay is based on the biochemical conversion of [^{3}H] L-arginine to [^{3}H] L-citrulline by NOS. Briefly control and test compounds treated cells were washed in PBS, harvested and centrifuged for 2 min to pellet the cells. The cells were resuspended in 1x homogenization buffer and sonicated briefly. Cells were centrifuged, the supernatant was separated and the resulting protein concentration was adjusted to 5-10 mg/mL. The eNOS activity was measured using the Cayman Chemicals NOS activity assay kit (Cayman Chemical Company, MI, USA).

Measurement of reduced glutathione (GSH)

Briefly, monochlorobimane stock (100 μM) was added to endothelial cells treated with test compounds. After 30 min, the medium was collected for medium GSH measurement. Cells were washed with PBS and harvested in 1000 μL of 1% sodium dodecylsulfate (SDS) in 50 mM Tris buffer (pH 7.5), sonicated and the aliquots (100 μL) of supernatants were read in triplicate with an excitation wavelength of 380 nm and an emission wavelength of 470 nm (Wu *et al*., 2002).

Implantation of subcutaneous MG pump

Subcutaneous pumps (Alzet® 2ML4) were implanted in all rats (Fig. 1-9) delivering either saline (2.5 µl/h) or MG (1.2 mg/h) by continuous infusion for 28 days. BSO, a GSH synthesis inhibitor and ALA, a MG scavenger were administered in drinking water (30 mg/kg) for 28 days. The site for s.c. implantation of ALZET pumps in rats will be on the back, slightly posterior to the scapulae. The rats were anesthetized with isoflurane, the skin over the implantation site was shaved and washedand sterilized with povidone iodine solution. A suitable mid-scapular incision was made adjacent to the site chosen for pump placement. A hemostat was inserted into the incision by opening and closing the jaws of the hemostat, the subcutaneous tissue was spread to create a pocket for the pump. Care was taken to make sure that the pump was not rest immediately beneath the incision, which could interfere with the healing of the incision. A filled pump was inserted into the pocket. The wound was closed with wound clips or sutures. Post-operative recovery was observed closely for the first 1-2 h. The wound site was observed everyday post-op for signs of infection and inflammation. Any infection wase treated with locally applied antibiotics.

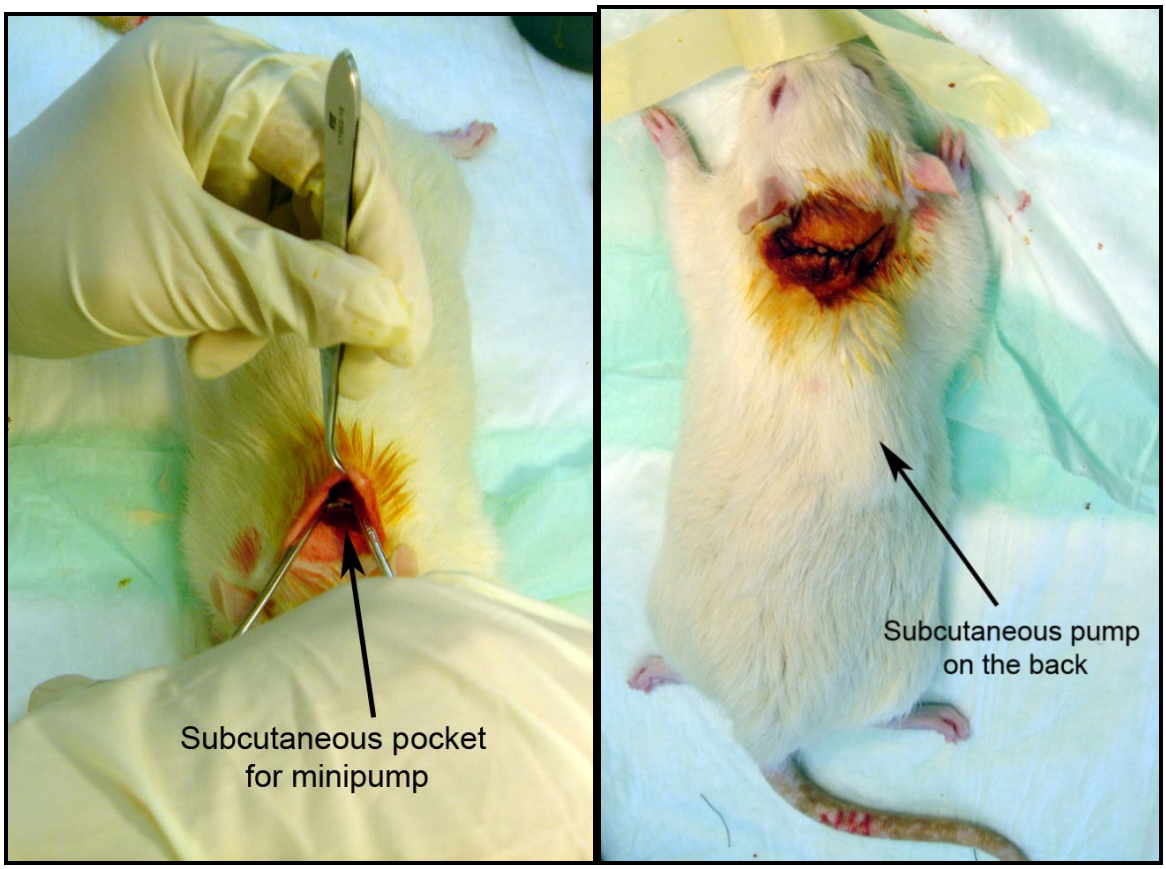

Figure 1-10 Surgical implantation of an osmotically driven infusion minipump filled with methylglyoxal. An incision was made on the back under aseptic conditions, just behind the ears in an anesthetised rat under isoflurane anaesthesia. A subcutaneous pocket was created in the fascia and the pump was inserted, followed by closure of the incision with surgical sutures. The rat was under buprenorphine (0.05mg/kg) analgesia pre- and post-surgically.

Oral Glucose Tolerance Test (OGTT)

After an overnight fast, basal blood sample was drawn through the tail vein in ice cold EDTA containing Eppendorf tubes. A bolus dose of glucose (1g/kg) was given through oral administration and blood samples were collected at 0, 15, 30, 60, 90 and 120 min from the tail vein and analyzed for glucose using glucose assay kit (BioAssay Systems, Hayward, CA,

USA) and insulin using rat insulin ELISA kit (Mercodia Rat Insulin ELISA, Uppsala, Sweden).

Insulin tolerance test (ITT)

After an overnight fast, the rats received an insulin injection (1U/Kg, i.v.). Blood samples were collected in EDTA containing tubes. Plasma was separated by centrifugation and plasma glucose levels were measured before and after 5, 10, 15, 30, 60 and 90 min using glucose assay kit.

Insulin release from freshly isolated pancreatic islets

The pancreatic islets were freshly isolated from SD rats as described by others (Shewade *et al*., 1999). Briefly, rats were anesthetised with isoflurane, the pancreatic duct was cannulated and injected with ~ 7 ml of ice-cold collagenase (Type IV, Worthington, 5 mg/ml) solution in Krebs buffer. The pancreas was cut out, washed in Krebs buffer and finely chopped into small pieces. The tissue was digested with 20 ml collagenase (Type IV) for 20 min at 37 °C in a shaker bath. The digestion was stopped by addition of 20 ml of calcium-free ice-cold Krebs buffer. The digested tissue was then centrifuged at 1000 rpm for 10 min and washed twice with Krebs buffer. The cell suspension was transferred into a Petri dish. Islets were separated under a dissection microscope. The identity of islets was confirmed under a high power microscope. For each treatment 10-20 islets were used in Eppendorf tubes. The islets were incubated for 30 min at 37°C in Krebs buffer containing either 5mM or 20mM glucose. The incubation medium was collected and analyzed for released insulin with insulin assay kit.

Terminal deoxynucleotidyl transferasedUTP nick end labelling (TUNEL) assay

The TUNEL assay was done using TUNEL assay kit (Roche Diagnostics, Indianapolis, USA). In brief, paraformaldehyde- fixed and optimal cutting temperature (OCT) compound-embedded sections of pancreas were cut into 4μM on glass slides. The sections were washed twice with PBS. After permeation with 0.1% Triton X-100 for 5 min and two washes with PBS, the tissue sections were incubated with blocking solution (3% H_2O_2 in methanol) for 10 min. After washing with PBS the slides were incubated with TUNEL reagent for 1h in dark and then converter POD (horse-radish peroxidise) for 30 min. After rinsing with PBS, the 3,3'-diaminobenzidine (DAB) reagent was added; the sections were mounted under glass cover slips and analyzed under light microscope.

DNA fragmentation assay

DNA was isolated from pancreas using DNA extraction kit (R & D Systems, MN, USA). Equal amount and concentration (1μg) of DNA was loaded on 1% agarose gel and run at 100V for 2h. The gel was visualized on Syngene bio-imaging system.

Real time Quantitative PCR (RT-PCR)

RNA was isolated from the pancreas using RNA isolation kit (Qiagen sciences, Germantown, MD, USA). The total RNA was reverse-transcribed in triplicate using RevertAid™ H Minus M-MuLV reverse transcriptase (MBI, Fermentas, Burlington, ON, Canada) in the presence of 5x RT buffer (MBI, Fermentas), Random primer (Invitrogen), dNTP mixture (Amersham Pittsburgh, PA, USA) at 42°C for 50 min, followed by 72°C for 10 min. The pre-designed primers for Pdx-1 and Maf-A were from Qiagen sciences,

Germantown, MD. The real-time PCR was carried out in an iCycler iQ apparatus (Bio-Rad Hercules, CA, USA) associated with the ICYCLER OPTICAL SYSTEM software (version 3.1) using SYBR Green PCR Master Mix (Bio-Rad). All PCRs were triplicated and performed in 96-well optical-grade PCR plates and run for 45 cycles at 95°C for 20 s, 62°C for 1 min, and 72°C for 30s. After cycling, melting curves of the PCR products were acquired by stepwise increase of the temperature from 62° to 95°C.

CHAPTER 4

METHYLGLYOXAL PRODUCTION IN VASCULAR SMOOTH MUSCLE CELLS FROM DIFFERENT METABOLIC PRECURSORS

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Abstract

Methylglyoxal (MG), a metabolic byproduct, reacts with certain proteins to yield irreversible advanced glycation endproducts (AGEs) and increases oxidative stress that causes the pathophysiological changes in diabetes, hypertension and aging. Although MG production from glucose has been well documented, the contribution of other intermediates of different metabolic pathways to MG formation is far less known. Our aim was to determine and compare the formation of MG, MG-induced AGE, N^{ε}-carboxyethyl-lysine (CEL), inducible nitric oxide synthase (iNOS), nitric oxide and peroxynitrite from different metabolic precursors in cultured rat aortic vascular smooth muscle cells (VSMCs). High-performance liquid chromatography was used to determine MG levels whereas nitrite plus nitrate, indicators of nitric oxide production, and peroxynitrite levels were measured with specific assay kits. CEL and iNOS were detected using immunocytochemistry. There was a concentration-dependent increase in MG levels in VSMCs after 3 h incubation with 5, 15 and 25 mM of D-glucose, fructose or aminoacetone. Aminoacetone produced a 7 fold increase in MG levels above the basal value followed by fructose (3.9 fold), D-glucose (3.5 fold), acetol (2.8 fold) and sucrose (2.3 fold) after a 3h incubation with 25 mM of each precursor. L-glucose, 3-O-methylglucose, and mannitol had no effect on MG production. All precursors, except L-glucose, 3-O-methylglucose and mannitol, increased CEL. Aminoacetone, D-glucose and fructose significantly increased iNOS, nitrite/nitrate and peroxynitrite levels. In conclusion, aminoacetone is the most potent precursor of MG production in VSMCs, followed by fructose and D-glucose. This could have important implications in relation to high dietary fructose and protein intake.

Keywords: methylglyoxal, advanced glycation end products, fructose, inducible nitric oxide

synthase, peroxynitrite

1. Introduction

A number of highly reactive metabolic intermediates of glucose, fatty acids, and amino acids react with proteins, lipids, DNA and other molecules and alter their normal structure and/or function (Desai and Wu, 2007). Methylglyoxal (MG) is one such highly reactive dicarbonyl compound formed mainly in the glycolytic pathway non-enzymatically (Thornalley, 1996; Kalapos, 1999) (Fig. 4-1A, B). Other sources of MG include intermediates of protein metabolism, such as aminoacetone (Deng *et al*., 1998), and fatty acid metabolism, such as acetone and acetol (Casazza *et al*., 1984) (Fig. 4-1A, B). The enzymes catalyzing conversion of aminoacetone and acetone/acetol to MG are semicarbazide-sensitive amine oxidase (SSAO) (Yu *et al*., 2003) and acetone mono-oxygenase/acetol monooxygenase (Casazza *et al*., 1984), respectively. SSAO is found in vascular smooth muscle cells (VSMCs) and the plasma (Yu *et al*., 2003).

MG reacts oxidatively with arginine or lysine residues of proteins and forms irreversible advanced glycation endproducts (AGEs) such as argpyrimidine (Shipanova *et al*., 1997), N^{ε}-carboxymethyl-lysine (CML) (Reddy *et al*., 1995), and N^{ε}-carboxyethyl-lysine (CEL) (Ahmed *et al*, 1997). Along with 3-deoxyglucosone and glyoxal, MG is a major source of intracellular and plasma AGEs (Vlassara *et al*., 1994). Elevated MG levels and AGEs such as CEL and CML are markers of carbonyl overload, oxidative stress and the resultant damage in aging and diabetes (Vlassara *et al*., 1994). An increase in glucose leads to increased formation of MG as occurs in diabetes (Thornalley, 1996; Vlassara *et al*., 1994). The serum concentration of MG increases in patients with type 1 or type 2 diabetes (Wang *et al*., 2007;

McLellan *et al*., 1994). We have recently shown that MG attaches to the internal arginine in the β-chain of insulin (Jia *et al*., 2006) and significantly reduces its capacity to stimulate [^{3}H]-2-deoxyglucose uptake by 3T3-L1 adipocytes and L8 skeletal muscle cells, compared with native insulin. These structural and functional abnormalities of insulin may contribute to the pathogenesis of insulin resistance (Jia *et al*., 2006). Our studies have also shown that as spontaneously hypertensive rats (SHR) developed hypertension there was an increase in MG levels in the plasma and aorta in an age-dependent fashion. No difference was observed in blood glucose levels between SHR and normotensive Wistar-Kyoto (WKY) rats (Wang *et al*., 2005; Wang *et al*., 2007).

We have shown earlier that MG activated nuclear factor kappaB p65 (NF-κB p65) and increased oxidative stress in rat VSMCs rats (Wu and Juurlink, 2002). MG induced inducible nitric oxide synthase (iNOS) and increased formation of peroxynitrite ($ONOO^-$), a highly reactive oxidant, that was inhibited by the NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME) (Chang *et al*., 2005; Wang *et al*., 2006). Increased peroxynitrite has been found in type 1 diabetes (Pacher *et al*., 2007). Superoxide, hydrogen peroxide, and peroxynitrite activate NF-κB which triggers inflammation and proliferative responses in VSMCs (Hoare *et al*., 1999).

Glucose, the principal carbohydrate nutrient in the body, is also the most important contributor to the formation of glycated proteins (Desai and Wu, 2007; Thornalley, 1996) (Vlassara *et al*., 1994). Fructose, another monosaccharide consumed in our diet, is also a precursor of MG (Wang *et al*., 1996). Sucrose from the diet is converted into glucose and fructose. Metabolic intermediates such as glucose-6-phosphate form AGEs at a faster rate than glucose (Bucala *et al*., 1984).

Given the importance of endogenous MG level and the potential for MG production from various metabolic intermediates of carbohydrates, proteins, and fatty acids (Fig. 4-1B), it is imperative to characterize the transformation capability of different metabolic precursors to MG in the cells. The production of MG from glucose has been well documented and quantitated (Thornalley, 1996; Kalapos, 1999; Vlassara *et al*., 1994). Unfortunately, production of MG from other metabolic precursors, such as aminoacetone, acetol, or sucrose has not been systematically and directly examined or quantitatively compared with that from glucose. Our main aim was to determine and compare the formation of MG from different precursors (Fig. 4-1A, B) in VSMCs to provide fundamental information for cellular MG production. MG-induced CEL, iNOS, NO and peroxynitrite in the presence of different metabolic precursors were further investigated in VSMCs.

2. Methods

2.1 Vascular smooth muscle cell culture

Rat thoracic aortic smooth muscle cell line (A-10 cells) was obtained from American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37° C in a humidified atmosphere of 95% air and 5% CO_2, as described in our previous studies (Chang *et al*., 2005; Wang *et al*., 2006) . A-10 cells were seeded either in 100 mm dishes for MG measurement or in 96-well plates for other assays, with an equal amount of cells (10^6/ml) in each well, and cultured to confluence. For immunocytochemistry staining, cells were seeded on cover glass slides (2×10^6/ml). Cells were starved in FBS-free DMEM for 24 h before exposure to different metabolic precursors.

2.2 MG measurement

MG was measured by a specific and sensitive high-performance liquid chromatography (HPLC) method (Wang *et al*., 2005; Wang *et al*., 2006). MG was derivatized with *o*-phenylenediamine (*o*-PD) to form the quinoxaline product, 2-methylquinoxaline, which is very specific for MG (Wang *et al*., 2005; Wang *et al*., 2007). After the specified incubation time of the cultured cells with precursors such as glucose and fructose, the culture medium was aspirated completely and the cells were washed twice with phosphate buffered saline (PBS). The cells were scrapped and cell pellets were resuspended in ice-cold PBS, and lysed over ice by sonication (5 s, three times). The sample was then incubated on ice for 10 min with ¼ volume of 1 N perchloric acid (PCA) and centrifuged (12,000 rpm, 15 min) to remove the PCA-precipitated material. The supernatant was supplemented with 10 mM *o*-PD and incubated for 3 h at room temperature. The 2-methylquinoxaline and underived 5-methylquinoxaline, which was added to the samples as the internal standard, were quantified on a Hitachi D-7000 HPLC system (Hitachi, Ltd., Mississauga, ON, Canada) via Nova-Pak® C18 column (3.9×150 mm, and 4 μm particle diameter, MA, USA).

2.3 Measurement of peroxynitrite

The formation of peroxynitrite was determined by a sensitive dicholorofluorescin (DCFH) assay as described previously (Wang *et al*., 2006). Briefly, cells were loaded with a membrane-permeable, nonfluorescent probe 2',7'-dichlorofluorescin diacetate (CM-H2DCFDA, 5 μM) for 2 h at 37°C in FBS-free DMEM in the dark. After washing with PBS 3 times, cells were treated with or without different substrates for 3 h, and finally subjected to detection. Once inside the cells, CM-H2DCFDA becomes membrane-impermeable DCFH2 in

the presence of cytosolic esterases, and is further oxidized by peroxynitrite to form the fluorescent oxidized dichlorofluorescein (DCF). The probe has high reactivity with peroxynitrite and its products $CO_3^{\cdot -}$ and $NO_2^{\cdot}$ but is not entirely specific for it. It also has low reactivity for hydrogen peroxide and even lower for superoxide (Wardman, 2007). The fluorescence intensity was measured with excitation at 485 nm and emission at 527 nm utilizing a Fluoroskan Ascent plate reader (Thermo Labsystem) and Ascent software, and expressed in arbitrary units.

2.4 Measurement of nitrite and nitrate

Cells were incubated with different substrates for 3 h and then washed with PBS. The supernatant was used for the measurement of nitrite and nitrate with a fluorimetric assay kit (Calbiochem) based on the Greiss reaction. The assay is based on the enzymatic conversion of nitrate to nitrite by nitrate reductase followed by the addition of 2,3-diaminonaphthalene, which converts nitrite to a fluorescent compound. Fluorescence intensity measurements of this compound accurately determine the nitrite (NO_2) concentration (excitation max.: 365 nm; emission max.: 450 nm).

2.5 Immunocytochemistry

A-10 cells were seeded on glass cover slips followed by incubation with different substrates for 3 h, and subjected to iNOS and CEL staining. As described previously [18], the treated cells were fixed in 4% paraformaldehyde for 30 min at room temperature and washed twice with 0.01 N phosphate buffered saline (PBS). After permeation with 0.1% Triton X-100 for 5 min and two washes with PBS, the cells were incubated with normal goat serum (diluted

1:30 in 0.1 N PBS) for 1 h to block non-specific binding sites. After shaking off the goat serum the slides were incubated with iNOS antibody (1:500; BD Transduction Laboratories) and CEL antibody (1:100; a generous gift from Novo Nordisk, A/S, Denmark) overnight at room temperature. Cells were washed twice in PBS (0.01 N) for 5 min and incubated with secondary fluorescein isothiocynate (FITC) conjugated anti-CEL and texas red conjugated anti-iNOS antibodies (Molecular Probes) for 2 h. After washing thrice with PBS the slides were mounted in glycerol:PBS (3:7), coverslipped and observed under a fluorescence microscope. Staining intensity was quantified using the Metapmorph image analysis software (*v*. 7, Molecular Devices). Slides from four different experiments were analyzed with 5 fields per slide observed and averaged.

2.6 Materials

The substrates used in this study (Fig. 1A), D- and L-glucose, mannitol, fructose, sucrose, 3-O-methylglucose (3-OMG) and acetol were purchased from BDH. Aminoacetone was synthesized and characterized in our laboratory as described previously (Kazachkov *et al*., 2005). All cell culture reagents were purchased from GIBCO.

2.7 Data analysis

Data are expressed as mean ± SEM and analyzed using one way ANOVA and post-hoc Dunnett's test where applicable. $P<0.05$ was considered statistically significant when group difference was compared.

3. Results

3.1 Generation of MG from different metabolic precursors

Cultured VSMCs were incubated with D-glucose, fructose, and aminoacetone at different concentrations for 3 h. The 3 h incubation time was based on our previous observation of a time-dependent production of MG after incubation of VSMCs with fructose that reached a peak at 3 h (Wang *et al*., 2006). All three precursors induced a concentration-dependent increase in MG levels (Fig. 4-2A). The greatest increase was observed with aminoacetone whereas fructose and D-glucose exhibited similar potency in generating MG (Fig. 4-2A, B). Even at 5 mM the amount of MG produced by aminoacetone is 3.6 fold more than that produced by an equimolar concentration of D-glucose and 3.9 fold above the baseline levels of MG (Fig. 4-2A).

In subsequent comparative experiments, in addition to D-glucose, fructose, and aminoacetone, acetol and sucrose also produced significant amount of MG in cultured VSMCs when 25 mM of each substrate was incubated for 3 h (Fig. 4-2B). However, L-glucose, 3-OMG and mannitol at the same concentration of 25 mM did not induce significant production of MG after 3 h incubation of VSMC (Fig. 4-2B).

3.2 Effect of different MG precursors on CEL formation

The levels of the MG-induced AGE, CEL, were increased in VSMCs after incubation with aminoacetone, fructose, D-glucose, acetol and sucrose, each at 25 mM for 24 h (Fig. 4-3A). CEL staining was located mainly in the cytosol of the cell with all precursors except aminoacetone, which showed CEL over the nuclear area also. The greatest increase was induced by aminoacetone, followed by fructose and D-glucose (Fig. 4-3B). Acetol and sucrose

caused less increase in CEL formation at a 25 mM concentration incubated for 24 h (Fig. 4-3B). L-glucose, 3-OMG and mannitol at a concentration of 25 mM did not cause CEL formation after 24 h incubation of VSMCs (data not shown).

3.3 Effect of different MG precursors on the generation of nitric oxide and peroxynitrite

Aminoacetone, fructose, and D-glucose all increased NO production, measured as the stable products nitrite+nitrate (Fig. 4-4A), and peroxynitrite levels (Fig. 4-4B) in VSMCs when 25 mM of each substrate was incubated for 3 h. Aminoacetone was the most potent inducer of NO and peroxynitrite production. No significant increases in nitrite+nitrate levels and peroxynitrite level were observed when VSMCs were treated for 3 h with 25 mM each of sucrose, acetol, L-glucose, 3-OMG, or mannitol (Fig. 4-4A & 4-4B).

3.4 Effect of MG precursors on inducible nitric oxide synthase expression

The staining intensity for iNOS was increased in VSMCs after incubation with aminoacetone, fructose, and D-glucose, each at 25 mM for 3 h (Fig. 4-5A). The greatest increase was induced by aminoacetone, followed by fructose and D-glucose (Fig. 4-5B). Acetol, sucrose, L-glucose, 3-OMG and mannitol at a concentration of 25 mM did not cause iNOS induction after 3 h incubation of VSMCs (data not shown).

The fold increase in MG, NO and peroxynitrite production has been shown in Fig. 4-6. This representation shows correlation of the magnitude of increase in nitrite+nitrate levels with the increase in MG levels after incubation with aminoacetone, fructose and D-glucose. In case of peroxynitrite, the ratio of peroxynitrite/MG showed correlation with the increase in MG levels obtained with aminoacetone, fructose and D-glucose (Fig. 4-6).

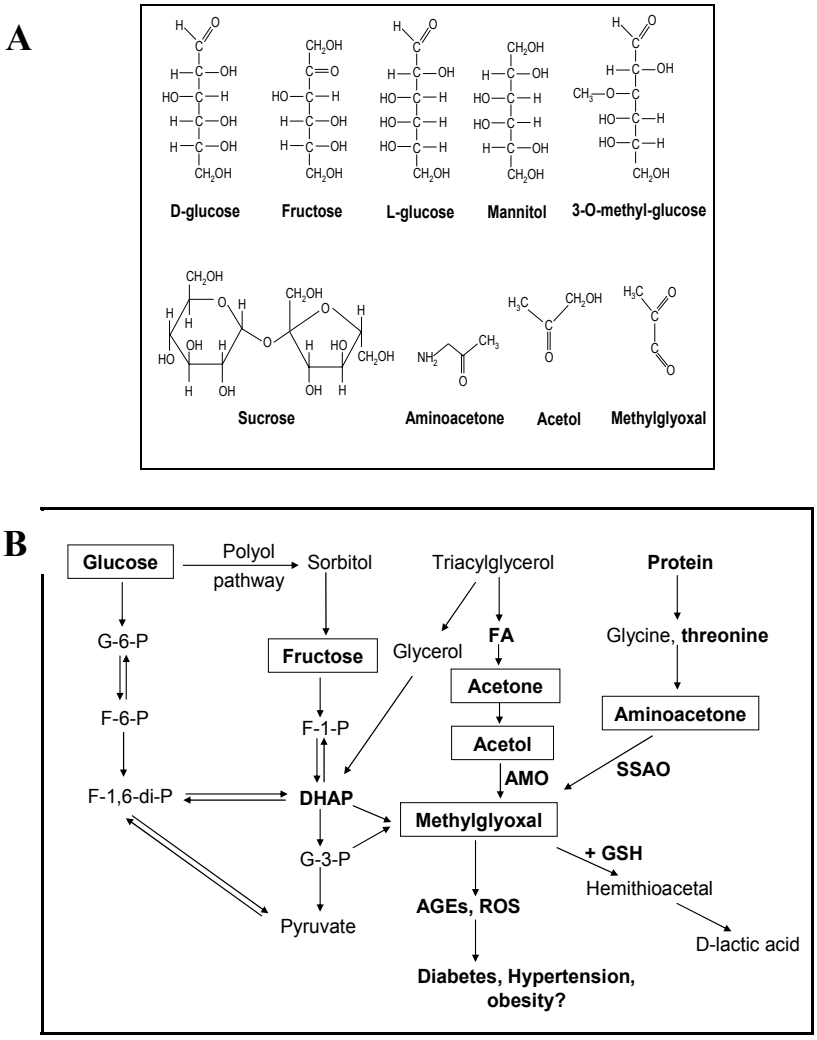


Fig. 4-1 A. Structures of methylgyoxal and various metabolic precursors used for the study. **B.** Pathways of methylglyoxal formation from different substrates.

AGEs – advanced glycation endproducts; AMO - acetone monooxygenase and acetol monooxygenase; DHAP – dihydroxyacetone phosphate; FA – fatty acid; F-1-P – fructose -1-phosphate; F-1,6-di-P – fructose -1,6-bisphosphate; F-6-P – fructose -6-phosphate; G-6-P – glucose -6-phosphate; G-3-P – glyceraldehyde -3-phosphate; GSH – reduced glutathione; ROS – reactive oxygen species; SSAO – semicarbazide-sensitive amine oxidase

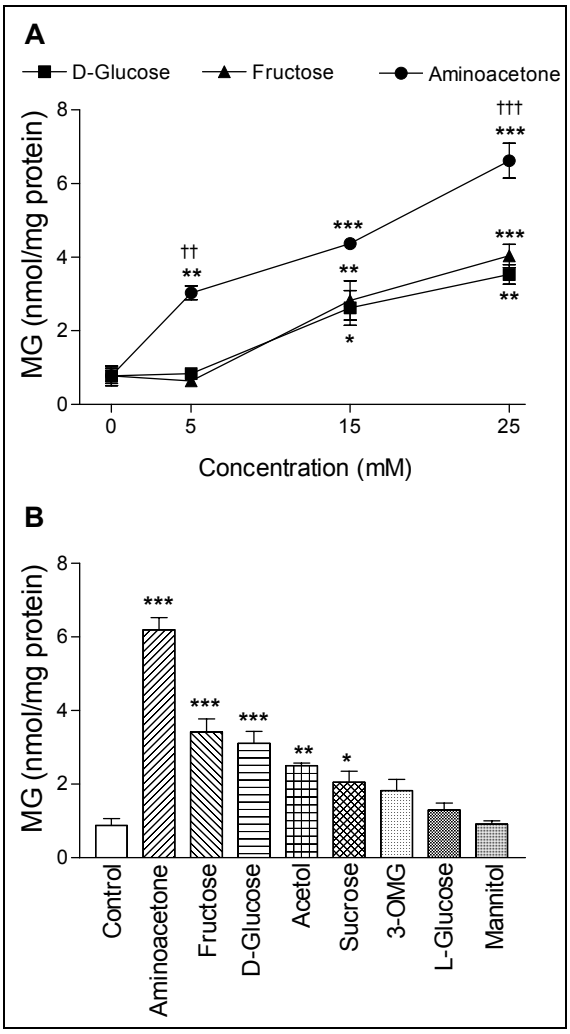


Fig. 4-2 **A.** Concentration-dependent production of methylglyoxal (MG) from different metabolic precursors in cultured rat aortic smooth muscle cells (A-10). The production of MG was determined after A-10 cells were incubated for 3 h with 5, 15 or 25 mM of precursors. n=3-4 for each group. *P<0.05, **P<0.01, ***P<0.001 *vs.* basal value. $^{\dagger\dagger}P$<0.01, $^{\dagger\dagger\dagger}P$<0.001 *vs.* fructose and D-glucose at same concentration. **B.** Different amounts of methylglyoxal (MG) are produced from different substrates in cultured rat aortic smooth muscle cells (A-10). The production of MG was determined after A-10 cells were incubated for 3 h with different substrates (25 mM each). n=6 for each group. *P<0.05, **P<0.01, ***P<0.001 *vs*. control

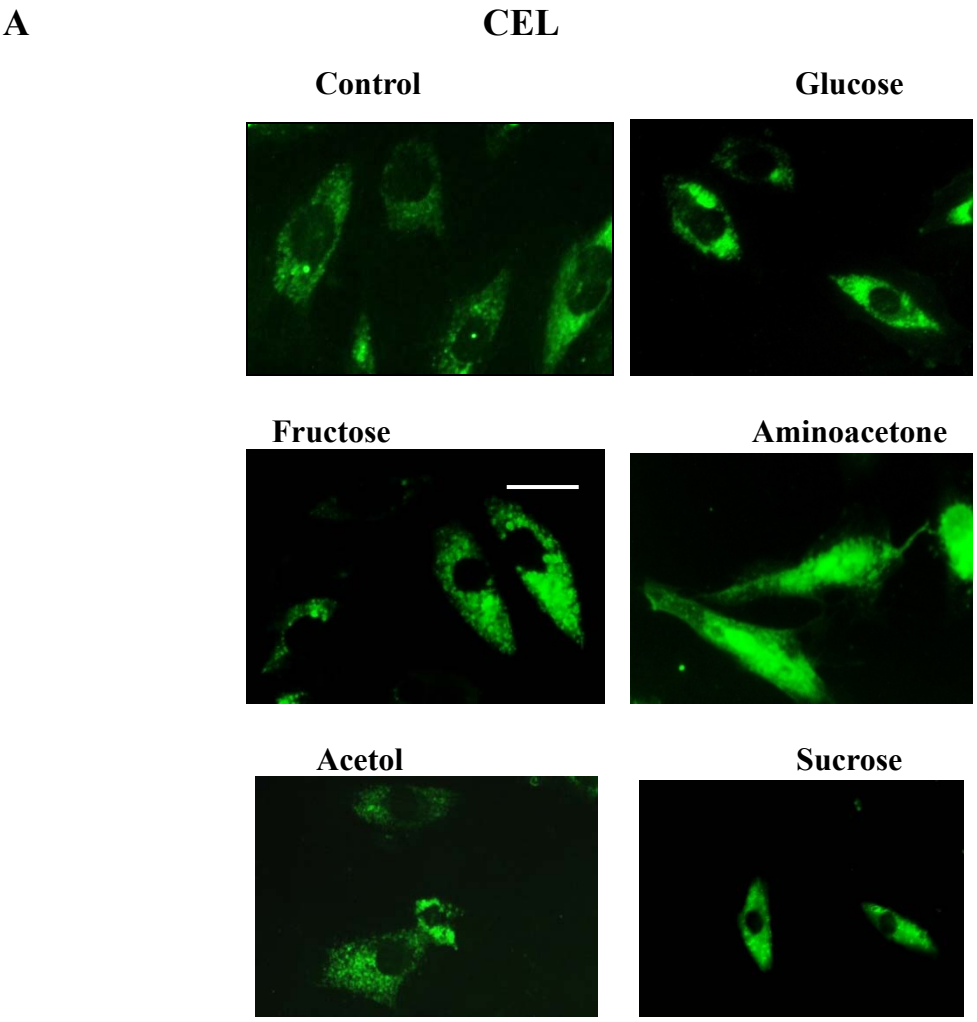


Fig. 4-3 Detection of methylglyoxal-induced advanced glycation endproduct, N^{ε}-carboxyethyl-lysine (CEL), in cultured rat aortic smooth muscle cells (A-10) after incubation

with different substrates (25 mM each) for 24 h. **A.** Immunocytochemistry was performed on fixed cells with specific CEL antibody and fluorescein isothiocynate (FITC) conjugated secondary antibody. Scale bar 10 μm. **B.** Staining intensity was quantified using the Metamorph imaging program (Molecular Devices). $^{**}P<0.01$ *vs.* control.

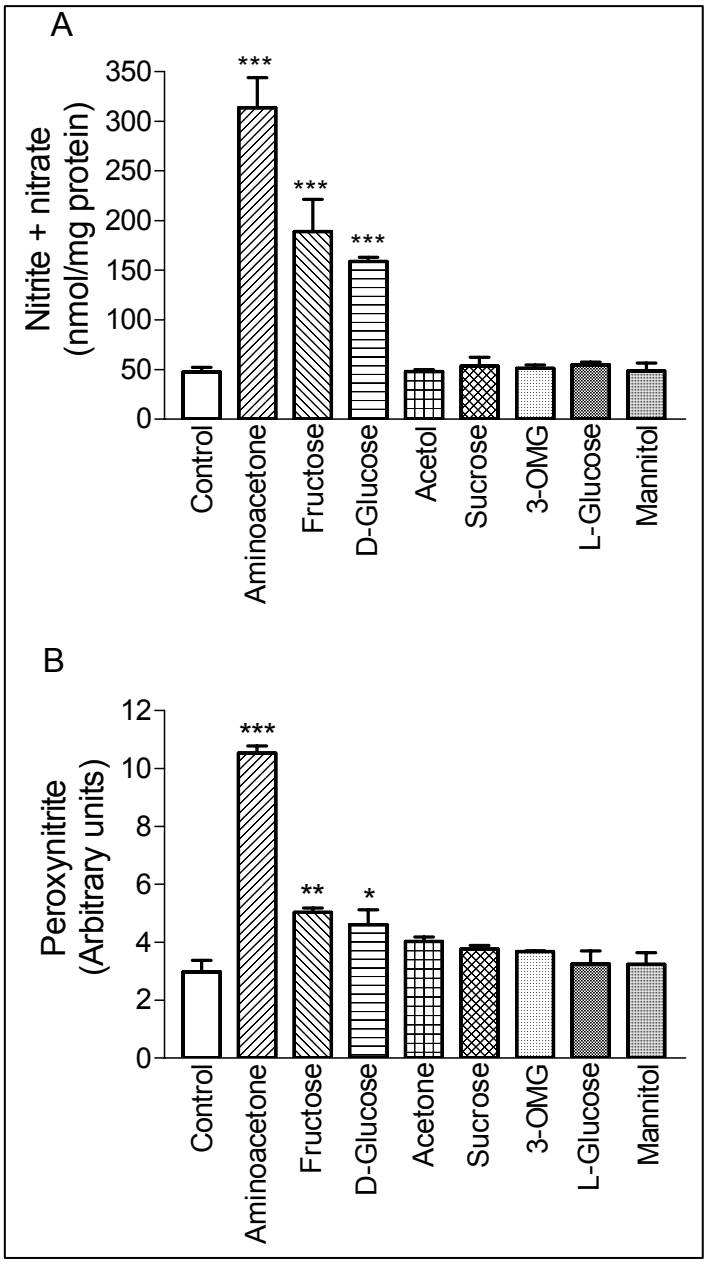
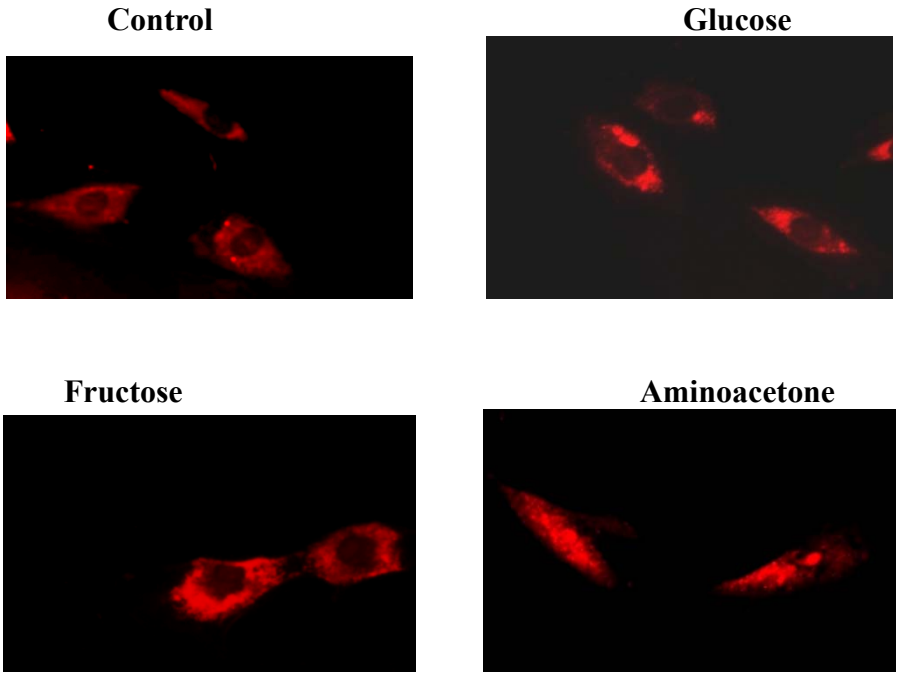


Fig. 4-4 Effect of different substrates on the production of nitrite plus nitrate (nitric oxide) (A) and peroxynitrite (B) in cultured rat aortic smooth muscle cells (A-10). The production of nitrite plus nitrate and peroxynitrite was determined after A-10 cells were incubated for 3 h with different substrates (25 mM each). n=3-6 for each group. *P<0.05, **P<0.01, ***P<0.001 *vs*. control.

A **iNOS**



B

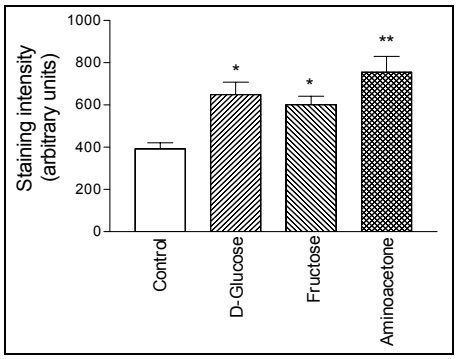


Fig. 4-5 Detection of inducible nitric oxide synthase (iNOS) in cultured rat aortic smooth muscle cells (A-10) after incubation with different substrates (25 mM each) for 3 h. **A.** Immunocytochemistry was performed on fixed cells with specific iNOS antibody and texas red conjugated secondary antibody. Scale bar 10 μm. **B.** Staining intensity was quantified using the Metamorph imaging program (Molecular Devices). *P<0.05, **P<0.01 *vs*. control.

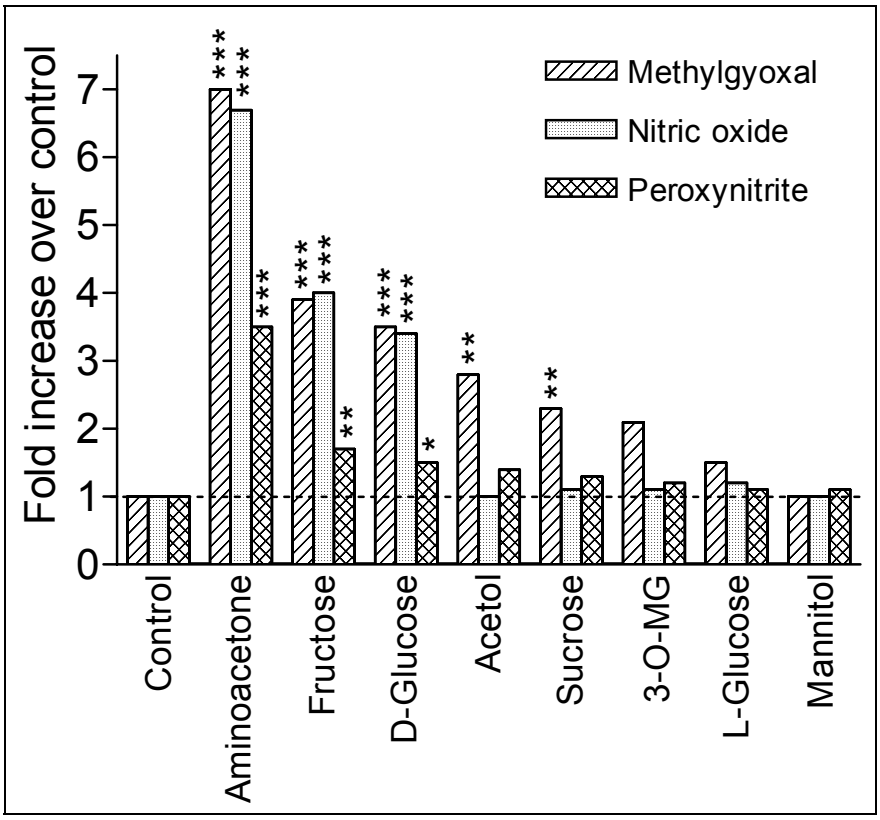


Fig. 4-6 Fold increase in methylglyoxal (MG), nitric oxide (nitrite plus nitrate) and peroxynitrite levels above basal values after incubation of cultured rat aortic smooth muscle cells for 3 h with different substrates (25 mM each). **P*<0.05, ***P*<0.01, ****P*<0.001 *vs.* control.

4. Discussion

We report major differences in MG produced in rat aortic smooth muscle cells incubated with different metabolic precursors derived from carbohydrates, proteins, and fatty acids. D-glucose, fructose, and aminoacetone induced concentration-dependent increase of MG. Aminoacetone, an intermediary product of L-threonine metabolism, produced the greatest amount of MG with a 7 fold increase above the baseline value (Fig. 4-6). Fructose and D-glucose at an equimolar concentration of 25 mM produced 3.9 and 3.5 fold increase in MG levels, respectively. Acetol and sucrose also produced significant MG whereas L-Glucose, 3-OMG and mannitol did not (Fig. 4-2B, 4-6). Importantly, aminoacetone, even at 5 mM, produced a significant increase in MG above the baseline levels whereas 5 mM D-glucose and fructose did not (Fig. 4-2A).

Aminoacetone, fructose, D-glucose, acetol and sucrose also caused MG-induced AGE, CEL, formation after 24 h incubation with VSMCs. MG reacts with cysteine, arginine and lysine residues of different proteins in a reversible or irreversible manner to form different endproducts. CEL, which is formed when MG reacts with lysine residues of proteins (Ahmed *et al*, 1997) has been detected in human lens proteins at a concentration similar to that of CML (Ahmed *et al*, 1997), which is one of the most frequently found MG-derived AGEs (Reddy *et al*, 1995). CEL increased with age in parallel with the concentration of CML and was formed in highest yields during the reaction of MG with lysine and protein and the authors suggested that CEL levels can provide an index of glyoxal and MG concentrations in tissues (Ahmed *et al*., 1997). In a recent study, the levels of MG-derived hydroimidazolone correlated with those of CML (Kilhovd *et al*., 2003), which in turn correlated with CEL formation (Ahmed *et al*., 1997). These AGEs can be detected immunohistochemically in

tissues (Reddy *et al*., 1995; Ahmed *et al*., 1997; Wang *et al*., 2005).

Aminoacetone, fructose and D-glucose also caused significant increase in NO and peroxynitrite formation and induced iNOS after 3 h incubation with VSMCs, whereas acetol, sucrose, L-glucose, 3-OMG and mannitol had no effect on NO and peroxynitrite formation and iNOS expression. It should be noted that that even though the probe 2',7'-dichlorofluorescin diacetate has high reactivity with peroxynitrite and its products $CO_3^{\cdot-}$ and $NO_2^{\cdot}$ (Warman 2007) it is not entirely specific for peroxynitrite. As mentioned in the methods this probe also has low reactivity for hydrogen peroxide and even lower for superoxide (Warman 2007). Previously we have shown that exogenous MG can induce iNOS and increase superoxide and peroxynitrite in VSMCs (Chang *et al*., 2005; Wang *et al*., 2006). In this study, the levels of MG correlate with nitrite+nitrate formed from different substrates (Fig. 4-6) and suggest a direct link between the amount of MG formed endogenously, and the magnitude of NO formation. One possible source of NO could be the increased levels of iNOS induced by aminoacetone, fructose and D-glucose as shown here (Fig. 4-5A, B) (Wang *et al*., 2006; Pacher *et al*., 2007; Hoare *et al*., 1999). Increased levels of iNOS are known to be associated with states of inflammation and immune reactions (Kaneki *et al*., 2007). Thus, MG can trigger an inflammatory reaction partly through induction of iNOS. Increased NO in turn can lead to increased formation of peroxynitrite, which can nitrosylate proteins and cause cellular damage (Pacher *et al*., 2007).

MG is detoxified via the glyoxalase system to form the inert D-lactate (Kalapos, 1999). Activity of the glyoxalase system is dependent on adequate levels of reduced glutathione (GSH) (Kalapos, 1999). Oxidative stress and hyperglycemia both lead to depletion of GSH (Wang *et al*., 2005; Chang *et al*., 2005; Park *et al*., 2003). Moreover, MG has been

shown to inactivate glutathione peroxidase and glutathione reductase (Chang *et al*., 2005; Park *et al*., 2003), which is required to recycle the oxidized glutathione (GSSG) back to GSH. This will impair the detoxification of MG and increase its half-life.

The high levels of MG, NO and peroxynitrite produced by aminoacetone may assume importance in conditions of increased amino acid metabolism, in particular increased L-threonine metabolism in people who are taking high amounts of protein such as athletes and body builders (Brandle *et al*., 1996) or in conditions of increased SSAO level such as hypertension and diabetes (Deng *et al*., 1998; Yu *et al*., 2003). L-threonine is metabolized to aminoacetone which can be deaminated to MG by SSAO, especially if the level of the antioxidant GSH is compromised as in cases of increased oxidative stress (Deng *et al*., 1998) (Vlassara *et al*., 1994; McLellan *et al*., 1994; Wu, 2002). Aminoacetone can be detected in the urine of normal people as well as patients with acute intermittent porphyria (Tschudy *et al*., 1964). However, it must be admitted that plasma levels of aminoacetone in the range of 25 mM that have been used in our *in vitro* study, have not been reported in the literature. We could not find any literature reports on the intracellular levels of aminoacetone thus limiting the significance of our findings regarding aminoacetone. Even so the potential of aminoacetone to form more MG and oxidative stress on a molar basis is a significant finding of our study.

Fructose produced equivalent amounts of MG, nitrite+nitrate and peroxynitrite as D-glucose (Fig. 4-6). In healthy people the plasma concentrations of fructose are low, around 0.13 mmol/L, compared to that of D-glucose, around 5 mmol/L, and it may not contribute to extracellular glycation as much as glucose (Kohlmeier *et al*., 2006). However, intracellular fructose is elevated in a number of tissues of diabetic patients in which the polyol pathway is

active and the concentrations of intracellular fructose and glucose are similar (Tomlinson *et al*., 1992). Plasma levels of fructose also increase in response to high fructose intake (Kohlmeier *et al*., 2006). No dietary fructose is needed since required amounts are modest and can be easily produced endogenously from glucose through the aldose reductase pathway (Tomlinson *et al*., 1992). Most fructose is consumed as refined sugar, such as sucrose, and through fruits and vegetables. In most Western societies the main source of fructose is high fructose corn syrup which is added to many industrial food products including ketchup and bread (Kohlmeier *et al*., 2006) and the daily intake of fructose maybe as high as 100 g (Ruxton *et al*., 1999). The glycemic index (GI) reflects the magnitude of increase in blood glucose levels after consuming a given carbohydrate. A GI of 55 or less is classed as low, between 56 and 69 as medium and more than 70 as high (http://www.glycemicindex.com/). The GI for a 50 g dose of fructose varies between 12 and 24 depending on the manufacturer (http://www.glycemicindex.com/). This is much lower than a GI between 85 and 111 for a 50 g dose of glucose. The GI for 50 g sucrose is between 58 and 65 (http://www.glycemicindex.com/). Due to the low GI of fructose diabetic patients are advised to eat lots of fresh fruits and vegetables (http://www.diabetes.co.uk/nutrition.html) (http://www.diabetes.org/nutrition-and-recipes/nutrition/healthyfoodchoices.jsp). This advice needs to be carefully re-evaluated in light of the equipotency of fructose, compared to D-glucose, in producing increased amounts of MG, NO and peroxynitrite (Fig. 4-6). Interestingly, in one study it was reported that in a population of vegetarians and omnivores plasma AGEs were higher (Krajcovicova *et al*, 2002). This was linked to a higher intake of fructose derived from a higher consumption of vegetables and fruit. Thus, it is likely that the increased formation of MG from fructose, as shown here, may substantially contribute to the

formation of intracellular AGEs, damage to cellular proteins and vascular complications (Mikulikova *et al*, 2008; Tokita *et al*, 2005).

As mentioned earlier, acetol is a precursor for MG formation. An equimolar concentration of acetol produced a 2.8 fold increase in MG levels compared to the 3.5 fold produced by D-glucose (Fig. 4-6). Acetone, which is converted to acetol, is one of the ketone bodies and increased plasma levels of acetone are encountered in situations of abnormally high lipolysis as in diabetic ketoacidosis (Owen *et al*., 1982), as well as during extended periods of fasting where there is increased fatty acid metabolism and with the ketogenic diet in children with epilepsy (Gasior *et al*., 2007). The ketogenic diet, composed of 80-90% fat and 10-20% carbohydrates and protein, is used for the treatment of drug-resistant seizures in children (Gasior *et al*., 2007). Mean serum acetone levels were 4 mM in children on the ketogenic diet and up to 8 mM in some of them (Musa *et al*., 2006). Acetone levels up to 8.9 mM were found in patients with diabetic ketoacidosis (Owen *et al*., 1982). Intracellular acetone/acetol levels are likely to be much higher than these values. Thus, there is a potential for increase in MG levels in these situations and indeed increased acetone (6.12 fold) and MG levels (1.67 fold) have been reported in subjects taking the Atkins diet which has a higher percentage of fat (Beisswenger *et al*., 2005).

Sucrose is the common sugar from sugar beets or sugar cane (Kohlmeier *et al*., 2006). Significant amounts are consumed with many sweet fruits. The sucrose in peaches may constitute as much as half of their dry weight. Sucrose is converted by the intestinal epithelium into glucose and fructose for absorption (Kohlmeier *et al*., 2006). Sucrose produced a relatively smaller increase in MG and CEL compared to D-glucose, and no significant increase in nitrite+nitrate or peroxynitrite. The result seems to indicate that sucrose

is not quickly converted to glucose and fructose in short period of time in the VSMCs.

L-glucose is the metabolically inert isomer of D-glucose (Kohlmeier *et al*., 2006) whereas 3-OMG is a non-metabolizable glucose analogue that is not phosphorylated by hexokinase. 3-OMG is used as a marker to assess glucose transport by evaluating its uptake within various cells and organ systems (Dringen *et al*., 1993). Mannitol is commonly used for its osmotic effect (Paczynski *et al*., 1997) and is not likely to be metabolized. As anticipated, L-glucose, 3-OMG and mannitol did not increase MG, CEL, nitrite+nitrate and peroxynitrite above the basal levels (Fig. 4-6). Thus, MG production is restricted to specific substrates.

The choice of 25 mM concentration is based on the observation that plasma glucose level in diabetic patients or STZ-induced diabetic rats (Cheng *et al*., 2001) can reach this high level and as such this concentration level can be used as a bench mark to compare the capabilities of other metabolic precursors on MG production.

In conclusion, the amount of MG formed in VSMCs depends a great deal on the nature of the substrate. The important finding of our study is that aminoacetone has the potential to form significant amounts of MG, CEL, NO and peroxynitrite even at low concentrations. Since aminoacetone is an intermediate of protein metabolism, the production of MG and peroxynitrite under conditions of increased protein catabolism is worthy of further investigation. Another key finding is that fructose, despite its low GI value compared to D-glucose, can form equal amounts of MG, CEL and induce oxidative stress as D-glucose. Increased fructose consumption, as seen in Western diets may be a health concern. The advice given to diabetic patients to eat lots of fruits, that contain fructose, needs to be carefully re-evaluated. Since currently the main tendency is to focus on glucose in terms of increased MG, AGEs and oxidative stress in conditions such as diabetes, it is important to consider the

contribution of other substrates such as fructose, acetol and AA to MG, AGEs and oxidative stress load. This is despite their lower concentrations and apparently no correlation to high glucose levels. Our findings also bear relevance to situations where the metabolic pathways are altered and the intracellular concentrations of metabolites such as fructose, aminoacetone and acetone/acetol may reach high levels.

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CHAPTER 5

METHYLGLYOXAL, PROTEIN BINDING AND BIOLOGICAL SAMPLES: ARE WE GETTING THE TRUE MEASURE?

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Abstract

Methylglyoxal (MG), a reactive metabolic byproduct and a precursor of advanced glycation endproducts (AGEs), is elevated in diabetes. In the body MG is free or reversibly or irreversibly bound (mostly with proteins). Variable plasma MG values have been reported. MG is commonly measured using high performance liquid chromatography. We tested several protocols on different biological samples, which resulted in significant differences in MG values measured in a given sample. The different values do not appear to be due to release and detection of bound MG under assay conditions. Protocols that provide consistent values of MG in biological samples are recommended.

Key words: Methylglyoxal, Perchloric acid, *o*-Phenylenediamine, HPLC

1. Introduction

Methylglyoxal (MG), an alpha-oxoaldehyde, is mainly formed from intermediates of glycolysis such as dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P) (Desai and Wu, 2007; Thornalley, 1996). It is also formed in lesser quantities from acetone during fatty acid metabolism and from threonine during protein metabolism (Desai and Wu, 2007; Thornalley, 1996). As a highly reactive electrophilic compound, MG is a major precursor of advanced glycation endproducts (AGEs). Glycation is a reaction between a free amino group of a protein and a carbonyl group of a reducing sugar, ultimately leading to the formation of advanced glycation endproducts (AGEs). MG reacts reversibly with cysteine residues to form hemithioacetal adducts, and with lysine and arginine residues to form glycosylamine residues (Lo *et al*., 1994). MG reacts irreversibly with lysine residues to form N_{ϵ}-(1-carboxyethyl)lysine (CEL) (Desai and Wu, 2007; Ahmed *et al*., 1997) and 1,3-di(N_{ϵ}-lysino)-4-methyl-imidazolium (MOLD) (Brinkmann *et al*., 1998) and with arginine to form N_{δ}-(4-carboxy-4,6-dimethyl-5,6-di-hydroxy-1,4,5,6-tetra-hydropyrimidine-2-yl)ornithine (THP) (Oya *et al*., 2000) and argpyrimidine (Shipanova *et al*., 1997). However, it has been reported that the major adduct formed *in vivo*, is MG-derived hydroimidazolone (MG-H) (Kilhovd *et al*., 2003) , which occurs as three structural isomers: N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1), 2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)pentanoic acid (MG-H2), and 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)pentanoic acid (MG-H3). AGEs are implicated in the pathological changes of diabetes mellitus and aging (Vlassara *et al*., 1994). Under normal physiological conditions, MG is kept at low levels by catabolism with glyoxalase I and glyoxalase II enzymes of the glyoxalase pathway that utilizes reduced glutathione (GSH) as a cofactor [2] but under hyperglycemic

condition, the levels of MG are high in plasma (McLellan *et al*., 1994; Thornalley *et al*., 1989; Beisswenger *et al*., 1999; Odani *et al*., 1999). To study the role of MG in various conditions it is critical to have a consistent protocol and a method of estimation that can give reproducible results when applied to different biological samples to measure MG levels.

In the literature, variable plasma levels of MG have been reported in humans plasma (McLellan *et al*., 1994; Thornalley *et al*., 1989; Beisswenger *et al*., 1999; Odani *et al*., 1999; Han *et al*., 2007) and rats (Nagaraj *et al*., 2002; Wang *et al*., 2007) and the different values of MG might be due to differences in protocol used for the preparation of samples. A number of methods have been developed for the measurement of MG in plasma and animal tissues (Bednarski *et al*., 1989; McLellan *et al*., 1992; Chaplen, *et al*., 1996; Hoffman *et al*., 1991; Sawicki *et al*., 1975; Ohmori, *et al*., 1989). At the center of each is a derivatization step to stabilize MG. The compounds which have been used to derivatize include 1,2-diamino-4,5-dimethoxybenzene (McLellan *et al*., 1992), ortho-phenylene diamine (*o*-PD) (Chaplen *et al*., 1996), o-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBOA) (Hoffman *et al*., 1991), dinitrophenylhydrazine (Sawicki *et al*., 1975; Ohmori *et al*., 1989) and 9-(3,4-diaminophenyl)acridine (DAA) (Mugo-Voloso *et al*., 2008). Dinitrophenylhydrazine may not be entirely specific for MG since it also reacts with intermediates from the glycolytic pathway to form the same osazones as MG (Ohmori *et al*., 1989). The most widely used and more specific method involves the derivatization of MG with 1,2-diaminobenzene derivatives, such as *o*-PD (Chaplen *et al*., 1996; Hoffman *et al*., 1991; Sawicki *et al*., 1975; Ohmori *et al*., 1989; Mugo-Veloso *et al*., 2008; Chaplen *et al*., 1998) and the quantification of the resulting quinoxaline with high performance liquid chromatography (HPLC). The high reactivity of MG might be a factor that makes it difficult for reliable and reproducible quantification from

sample to sample (Chaplen *et al*., 1998). It is believed that less than 1% of MG exists in a free form while more than 99% exists in a protein-bound form (Mugo-Veloso *et al*, 2008; Chaplen *et al*., 1998). To complicate matters, it has been proposed that the protein bound form can exist as an irreversibly bound pool or a reversibly bound pool (Chaplen *t al*., 1998; Ahmed *et al*., 2002). In this case the irreversibly-bound form remains stable under harsh assay conditions and therefore cannot be detected (Chaplen *et al*., 1998). Irreversibly-bound MG, characterized as AGEs, is detected by separate assays for AGEs (Ahmed *et al*., 2002; Nagaraj *et al*., 1996). The reversibly-bound MG is believed to be in dynamic equilibrium with free MG and can be measured (Fig.1). However, the reversibly bound MG compounds are unstable and are, therefore, possibly a source of error in assays (Chaplen *et al*., 1998). Perchloric acid (PCA) is used in the protocol to stop metabolic reactions in the sample and to precipitate proteins which are immediately removed from the sample before it is derivatized with *o*-PD (Chaplen *et al*., 1996; Hoffman *et al*., 1991; Sawicki *et al*., 1975; Ohmori *et al*., 1989; Mugo-Veloso *et al*., 2008; Chaplen *et al*., 1998). Chaplen *et al* (Chaplen *et al*., 1998) theorized that this will also precipitate the reversibly-bound MG adducts and remove them from the sample along with the proteins that are removed after centrifugation. According to the results obtained by Chaplen *et al.* (Chaplen *et al*., 1998), a longer incubation of the precipitated proteins, which will include the reversible MG adducts, will allow the acidic environment to free MG from its reversible binding and make it amenable to detection. Variations in the sample treatment protocol detected up to an amazing 100 to 1000 fold more MG from the same sample, when applied to cultured Chinese hamster ovary (CHO) cells (Chaplen *et al*., 1998). However, it is not known if similar variations in protocol would affect the amount of MG detected in other sample types such as plasma, body organs or tissues and other cultured cells such as vascular smooth

muscle cells (VSMCs). We tested the protocol variables on plasma and liver samples from Sprague-Dawley rats and on rat cultured aortic VSMCs. Our aim was to determine what impact the protocol variability had on the amounts of MG measured and establish optimum protocols for different sample types. Since 99% of MG is reported to exist in a protein bound form, we also prepared a MG (120 μM) - 1% bovine serum albumin (BSA, in phosphate buffered saline, PBS) and a MG (120 μM) – 1% liver homogenate in PBS solution to determine time-related reactivity, the degree of protein binding (reversible or irreversible) and the detectable amount of MG with different protocols.

2. Experimental

2.1 Preparation of samples

2.1.1 Preparation of plasma and liver samples. Six male SD rats, 12-13 weeks old, were obtained from, Charles Rivers, Quebec, Canada and treated in accordance with guidelines of the Canadian Council on Animal Care. The protocol was approved by the Animal Care Committee of the University of Saskatchewan. Animals were fed normal chow diet *ad libidum* and acclimatized for one week before the experiment. Blood was collected in EDTA tubes from anesthetized (thiopental sodium 100 mg/kg, intraperitoneal) normal SD rats. Plasma was separated by centrifugation (3000 g for 5 min). Liver was removed from normal SD rats and was frozen in liquid nitrogen. The liver sample was homogenized under liquid nitrogen in a Mikro-Dismembrator (B. Braun Biotech. Int., Bethlehem, PA, USA). The homogenized sample was reconstituted in PBS or sodium phosphate buffer (pH 4.5) and sonicated (30 s, three times). The same sample of plasma or liver was treated with designed protocols to study the impact of different protocols on the amount of MG measured. The experiment was

repeated a minimum of four times on plasma and liver samples from different rats.

2.1.2 Vascular smooth muscle cells: Rat thoracic aortic smooth muscle cell line (A-10 cells) was obtained from American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37° C in a humidified atmosphere of 95% air and 5% CO_2, as described previously (Dhar *et al*., 2008; Chang *et al*., 2005; Wang *et al*., 2006). A-10 cells were seeded in 100 mm dishes and were starved in FBS-free medium for 24 h prior to scrapping and collection. The cell pellet was reconstituted in PBS and sonicated (5 s, three times). The homogenate was used for MG level analysis and protein determination.

2.1.3 MG-BSA, MG-liver, MG-liver/EDTA and MG-PBS samples: MG (120 μM) was incubated for varying times ranging from 1 min to 24 h in an incubator (37º C) with 1% BSA in 1 N PBS (pH 7.4) solution (MG-BSA), 1% liver homogenate in 1 N PBS (pH 7.4) solution (MG-liver), 1% liver homogenate in sodium phosphate (pH 4.5) containing 50 μM EDTA (MG-liver/EDTA) or PBS alone (MG-PBS). The same sample was treated with different protocols. The experiment was repeated a minimum of four times on different samples.

2.2 Protocol variables:

2.2.1 Different samples were incubated with perchloric acid (PCA, 0.2 N or 0.45 N final concentration) for 10 min, 3 h or 24 h at room temperature, which has been described in results as PCA 10 min, 3 h or 24 h. The concentrations of PCA were chosen based on their use in previously published reports (McLellan *et al*., 1992; Hoffman *et al*., 1991; Chaplen *et al*.,

1998; Ahmed *et al*., 2002; Dhar *et al*., 2008; Wang *et al*., 2006; Randell *et al*., 2005). PCA was used to precipitate proteins from the sample and to inhibit metabolic reactions. The precipitated protein was removed from the samples by centrifugation (12,000 rpm for 10 min at 4º C).

2.2.2 *o*-PD (0.2 mM, 1 mM or 10 mM final concentration) was used as a thermodynamic trap to derivatize MG to form the stable 2-methylquinoxaline (Fig. 5-1). In case of PCA 10min, *o*-PD was added to the supernatant after removal of precipitated protein (McLellan *et al*., 1992; Chaplen *et al*., 1996; Chaplen *et al*., 1998; Ahmed *et al*., 2002; Dhar *et al*., 2008; Wang *et al*., 2006; Randell *et al*., 2005), while in case of PCA 3 h or 24 h it was added to the sample along with PCA and incubated at room temperature for 3 or 24 h (Chaplen *et al*., 1998). The sample was further centrifuged at 12,000 rpm for 10 min before adding the supernatant to the HPLC sample tubes. The concentrations of *o*-PD were chosen based on their use in previously published reports (McLellan *et al*., 1992; Hoffman *et al*., 1991; Chaplen *et al*., 1998; Ahmed *et al*., 2002; Dhar *et al*., 2008; Wang *et al*., 2006; Randell *et al*., 2005).

2.2.3 The incubation time was varied as described above.

2.3 Quantification of MG by high performance liquid chromatography (HPLC)

2.3.1 Method validation: Methylglyoxal was quantified on Hitachi D-7000 HPLC system (Hitachi D-7000 HPLC system (Hitachi, Ltd., Mississauga, ON, Canada) via Nova-Pak® C18 column (3.9×150 mm, and 4 µm particle diameter, MA, USA) using the external standard 2-

methylquinaxaline (2-MQ) method, by plotting the concentration of standard quinoxaline derivative (μM) as a function of peak area detected at 315 nm, corresponding to their UV absorption maxima. Regression equation and correlation coefficient, reported in Table 5-1, was calculated by least square method. The limit of detection (LOD), calculated as the amount of analyte required to obtain a signal to noise ratio of 2:1, was 3 μvolts The limit of quantification (LOQ), that is the lowest concentration required to yield a signal to noise ratio of 12, was 0.05 μM.

Method repeatability (inter and intra-assay) was evaluated by analyzing the derivatized samples 3 times in 3 days. The inter and intra-assay standard deviation of the methylglyoxal was consistently <3 (Table 5-2).

The recovery of the HPLC method was determined by recovery tests performed by adding four different concentrations of standard quinoxaline (2-MQ) derivative to samples before derivatization. The results (Table 5-3) showed recovery rates between 98-100%.

Table 5-1. Calibration data and LOQ and LOD of 2-methylquinoxaline

Compound	Linear range (μM)	Regression equation	Correlation coefficient	LOQ (μM)	LOD (μvolts)
2-methylquinoxaline	20%	y = 0.5634x-0.5464	0.9958	0.05	3

Table 5-2. Method of precision of 2-MQ in samples after derivatization with *o*-PD

Values of 2-MQ (μM) are Mean ± SEM.

Compound	Day 1	Day 2	Day 3	RSD (%) intra- assay (day 1)	RSD (%) (intra-assay)
2-methylquinoxaline	2.4 ± 0.03	2.3 ± 0.05	2.35 ± 0.02	1.7	1.41

Table 5-3. Recovery rates of the HPLC method for 2-MQ determination

Compound	Amount added (µM)	Amount found (µM)	% recovery (*n*=3)
2-methylquinoxaline	10	9.915	99.15

To determine recovery, two sets of samples (*n*=3 each) of rat plasma were prepared. One set of samples was analyzed as is and the second set was spiked with 10 µM of MG. The MG in both sets of samples was determined by HPLC. Calculation of recovery involved subtraction of the MG in the unadulterated plasma sample from that in the spiked sample. The recovery experiment was done using the internal standard 5-MQ. Recovery is shown in Table 5-4.

Table 5-4. Recovery rate of standard quinoxaline (2-MQ) after derivatization

Compound	Concentration (µM)	Peak area	Peak ratio (2-MQ/5-MQ)
2-methylquinoxaline	10	134252	1.164836
5-methylquinoxaline	100	115254	

Solvent B (100% methanol) was used for washing the column and lines. Solvent A (acetonitrile 20%) was kept at 100% for running the samples. Each sample run was for 30 min

with a flow rate of 1 ml/min. The whole series of samples in a single experiment was run in duplicate.

2.4 Chemicals

All chemicals were of analytical grade. Methylglyoxal, *o*-phenylenediamine (*o*-PD), ethylene diamine tetraacetic acid (EDTA), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), sodium phosphate dibasic (Na_2HPO_4), 2-methylquinoxaline (2-MQ), 5-methylquinoxaline (5-MQ) and perchloric acid (PCA) (ACS reagent grade) were purchased from Sigma Aldrich, Ontario, Canada. HPLC grade acetonitrile and methanol were purchased from EMD Chemicals Inc., Gibbstown, NJ, USA. Sodium metabisulfite ($Na_2S_2O_5$) was purchased from Alfa Aesar, A Johnson Matthey Company, MA, USA.

2.5 Statistical analysis

Data are expressed as Mean ± SEM and analyzed using one way ANOVA to compare differences between three or more values from the same sample subjected to different protocols. Student's unpaired *t*-test was used to compare differences between two values forming a pair. The *P* value was considered significant when it was less than 0.05 ($P<0.05$)

3. Results

3.1 Method validation: Regression equation and correlation coefficient, reported in Table 5-1, was calculated by least square method. The limit of detection (LOD), calculated as the amount of analyte required to obtain a signal to noise ratio of 2:1, was 0.01 μM. The limit of quantification (LOQ), that is the lowest concentration required to yield a signal to noise ratio

of 12, was 0.05 μM.

Method repeatability (inter- and intra-assay) was evaluated by analyzing the derivatized samples 3 times in 3 days. The inter- and intra-assay standard deviation of MG was consistently <3 (Table 5-2).

The recovery of the HPLC method was determined in two separate ways: 1) Plasma sample was divided into two. One was analyzed as the unadulterated sample after derivatization with *o*-PD. The other parallel plasma sample was spiked with a known amount of MG (10 μM) and was derivatized with *o*-PD. To calculate recovery the MG in the unadulterated plasma sample (without any externally added MG) was subtracted from the spiked sample (with 10 μM MG added). % recovery = (amount detected / amount added) x 100 (n = 3 each). As shown in Table 5-3 recovery was 99%.

2) Recovery of the external standard: Plasma sample was spiked with a known amount of external standard (2-MQ, 5 μM) and subjected to the entire HPLC protocol. 2-MQ detected in the spiked sample was then compared against the external standard (5 μM, used for calibration of standard curve) using the following equation. % recovery = [(2-MQ in spiked sample – 2-MQ in unspiked sample) / 2-MQ in external standard] x 100 (n = 3 each). As shown in Table 5-4 recovery was 102%.

3.2 Fig. 5-1 shows representative chromatograms of plasma, liver and VSMC samples.

3.3 Plasma samples: The same plasma sample treated with different protocols resulted in significant differences in the amount of MG measured (Fig. 5-2). The sample incubated with 0.45 N PCA consistently produced greater levels of MG than 0.2 N PCA (Fig. 5-2A). This

difference was remarkable when the sample was incubated with PCA (0.45 N) and *o*-PD (10 mM) for 24 h (Fig. 5-2A). When the PCA (0.2 or 0.45 N)-precipitated protein was incubated for 3 h or 24 h instead of 10 min, before removal by centrifugation, there was no significant difference in MG levels except in two instances (Fig. 5-2B). Incubation with 10 mM *o*-PD, as compared to 0.2 or 1 mM, also did not result in significantly different values of MG except in one instance where incubation of 10 mM *o*-PD and PCA (0.45 N) for 24 h produced almost double the value of MG in the same sample as compared with 0.2 and 1 mM *o*-PD (Fig. 5-2C).

3.4 Liver samples: The same liver sample treated with different protocols produced significant differences in the amount of MG detected (Fig. 5-3). Treatment with 0.45 N PCA consistently produced significantly higher values of MG than 0.2 N PCA (Fig. 5-3A). When the PCA (0.2 or 0.45 N)-precipitated-protein was incubated for 3 h or 24 h instead of 10 min, before removal by centrifugation, it gave a greater measure of MG in all instances (Fig. 5-3B). Incubation with 10 mM *o*-PD, as compared to 1 mM, produced significantly higher values of MG in most instances (Fig. 5-3C). 0.2 mM was not tried with liver samples.

3.5 Cultured VSMCs: The MG levels were also measured in A-10 cells with different protocols (Fig. 5-4). As with plasma and liver samples, 0.45 N PCA yielded significantly higher values of MG than 0.2 N PCA (Fig. 5-4A). Incubation of samples for 10 min or 3 h with 0.45 N PCA did not produce any difference in MG values. However, incubation of samples for 24 h with 0.45 N PCA resulted in higher values of MG, compared to 10 min or 3 h incubation (Fig. 5-4B). Incubation of samples with 1 or 10 mM *o*-PD did not produce

significant differences in values of MG detected in VSMCs (Fig. 5-4C).

3.6 Methylglyoxal-bovine serum albumin (MG-BSA) samples: In one group of experiments, MG (120 μM) was incubated with or without 1% BSA in 1 N PBS (pH 7.4) for different times (1 min to 24 h) and then MG levels were determined in these MG-BSA or MG-PBS (protein-free control) samples with different protocols. As shown in Fig. 5-5 the different protocols failed to produce significant differences in the amount of MG detected in MG-BSA samples. Incubation of PCA for 10 min, 3 h or 24 h resulted in equivalent values of MG (Figs. 5-5A, 5-5C). Similarly, incubation of the same MG-BSA sample with 1 or 10 mM *o*-PD did not result in any significant differences in MG values (Fig. 5-5B). Fig. 5C shows a time-dependent decrease in MG levels in the MG-BSA group. For example, after incubation of MG (120 μM) with 1% of BSA for 3 h and 24 h, the detectable MG was decreased to 66% and 22% of the value detected in MG-PBS, respectively (Fig. 5-5C). This indicates decreased free MG or increasing binding of MG to BSA with increasing time. In addition, incubation of MG-BSA with 0.45 N PCA for 10 min or 24 h did not produce significant differences in MG values. Based on previous results 0.2 N PCA and 1 mM *o*-PD were not tested with MG-BSA samples.

3.7 Methylglyoxal-liver homogenate (MG-liver) samples: Fig. 5-6A shows the amount of MG detected when 120 μM of MG was incubated for 3 or 24 h with 1% liver homogenate in 1 N PBS (pH 7.4) (MG-liver), 1% liver homogenate in sodium phosphate (pH 4.5) containing 50 μM EDTA (MG-liver/EDTA) or 1 N PBS (MG-PBS). The same sample treated with 0.45 N PCA for 10 min or 24 h did not produce any differences in the amount of MG detected (Fig. 5-

6A). *o*-PD (10 mM) was incubated for 24 h with all samples. Based on previous results 0.2 N PCA and 1 mM *o*-PD were not tried with MG-liver homogenate samples.

Fig. 5-6B shows that an incubation of the sample with 120 μM of MG for 3 or 24 h resulted in different levels of MG detected in the MG-PBS (control), MG-BSA, MG-liver and MG-liver/EDTA samples. In comparison with MG-PBS control, only 49% and 2% of MG can be detected in MG-liver samples after 3 h and 24 h incubation, respectively, while 65% and 19% of MG can be detected in MG-liver/EDTA samples and 66% and 24%, respectively, in the MG-BSA samples. This further suggests that decreased MG values reflect decreased free MG level with increased binding of MG to BSA or proteins in liver homogenate suspension with increasing incubation times. It also indicates possible degradation of added MG by liver enzymes in the MG-liver sample.

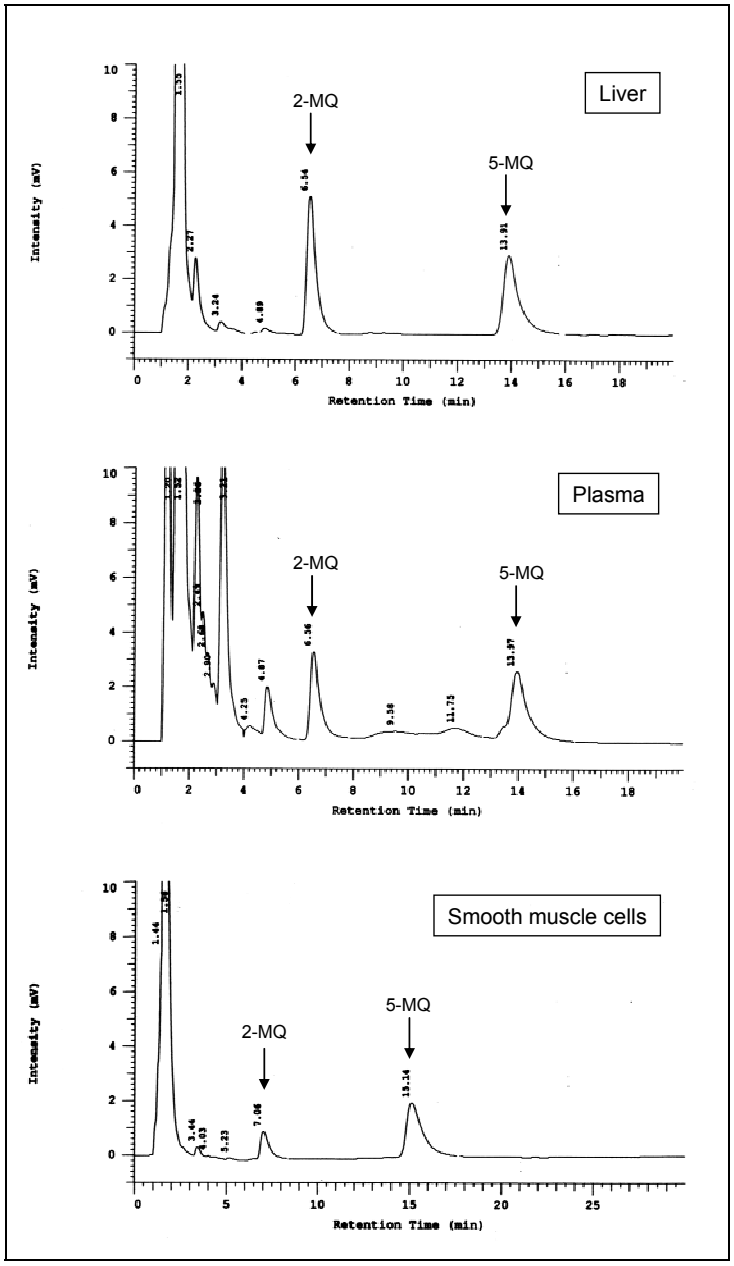

Fig. 5-1 Original chromatograms showing 2-methylquinoxaline (2-MQ) and 5-methylquinoxaline (5-MQ) peaks in samples of (A) liver, (B) plasma and (C) cultured rat aortic vascular smooth muscle (A10) cells. 2-MQ is a specific stable product formed by derivatization of methylglyoxal in the sample when the sample is incubated with *o*-phenylene diamine (*o*-PD). 5-MQ is the internal standard (10 μM).

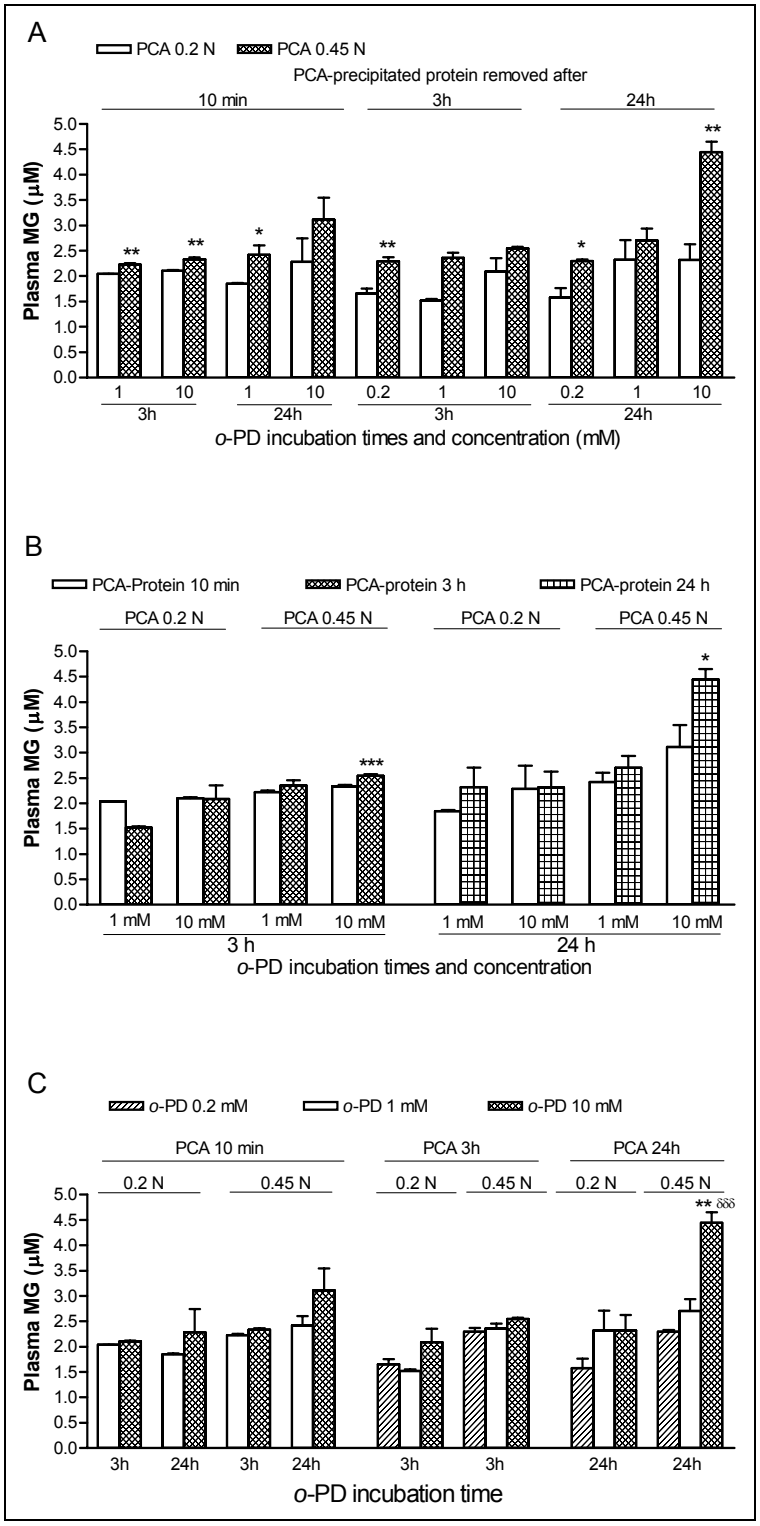

Fig. 5-2 Methylglyoxal (MG) levels in the plasma measured with different protocols. A. The

sample was acidified and deproteinized with 0.2 or 0.45 N perchloric acid (PCA). B. The PCA-precipitated protein was incubated in the sample for 10 min, 3 h or 24 h before removal by centrifugation. C. MG was derivatized by incubation with *o*-phenylenediamine (*o*-PD, 0.2, 1 or 10 mM) for 3 h or 24 h. n = 5-6 for each group. *P<0.05, **P<0.01, ***P<0.001 *vs*. corresponding paired value, $^{\delta\delta\delta}P$<0.001 *vs*. corresponding 0.2 mM value.

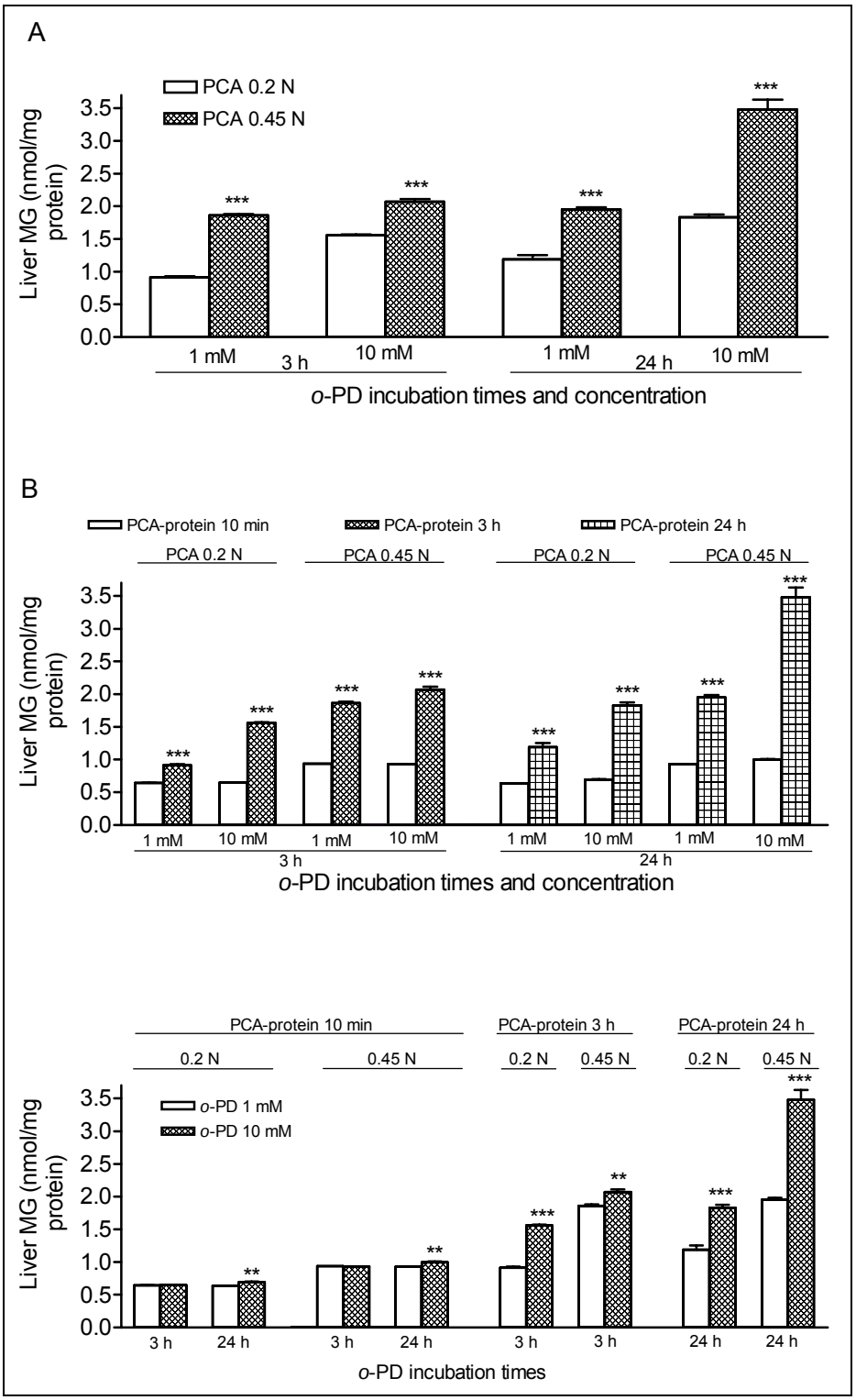

Fig. 5-3 Methylglyoxal (MG) levels in liver sample treated with different protocols. A. The sample was acidified and deproteinized with 0.2 or 0.45 N perchloric acid (PCA). B. The

PCA-precipitated protein was incubated in the sample for 10 min, 3 h or 24 h before removal by centrifugation. C. MG was derivatized by incubation with *o*-phenylenediamine (*o*-PD, 1 or 10 mM) for 3 h or 24 h. n = 4-5 for each group *P<0.05, **P<0.01, ***P<0.001 *vs*. corresponding paired value.

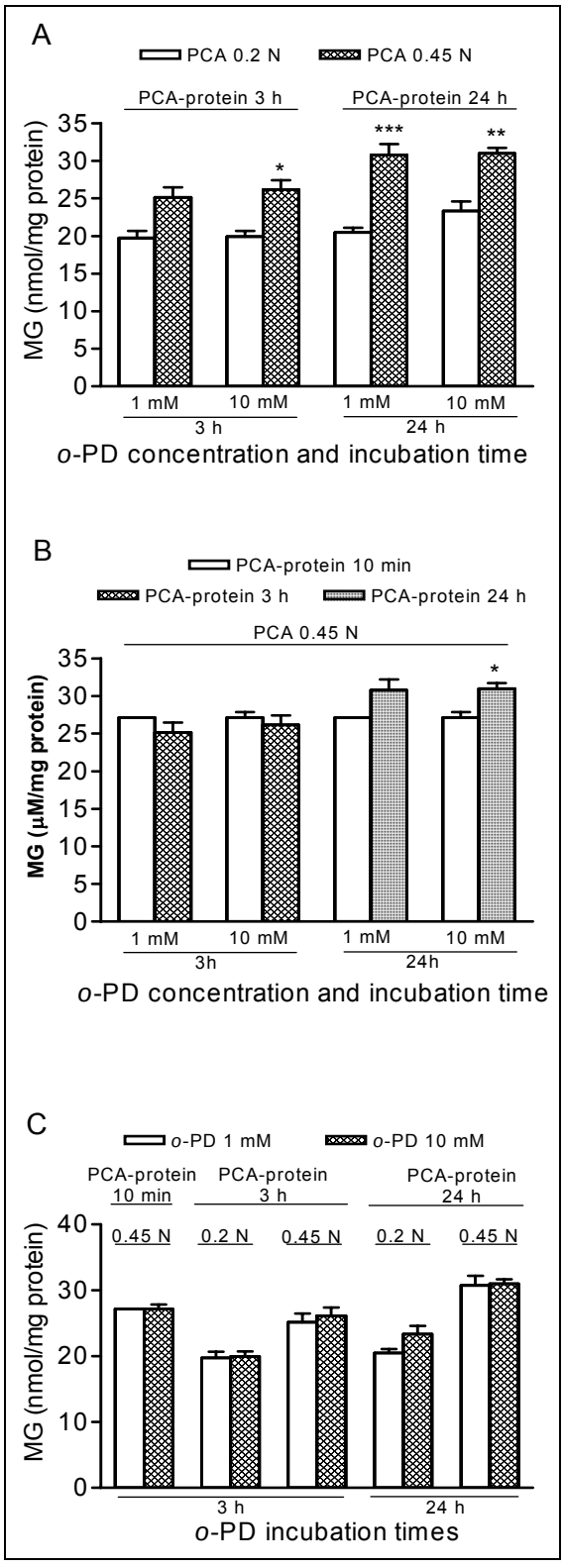

Fig. 5-4 Methylglyoxal (MG) levels in cultured vascular smooth muscle cells (VSMCs). A. The sample was acidified and deproteinized with 0.2 or 0.45 N perchloric acid (PCA). B. The

PCA-precipitated protein was incubated in the sample for 10 min, 3 h or 24 h before removal by centrifugation. C. MG was derivatized by incubation with *o*-phenylenediamine (*o*-PD, 1 or 10 mM) for 3 h or 24 h. n = 3-4 for each group. *P<0.05, **P<0.01, ***P<0.001 *vs*. corresponding paired value.

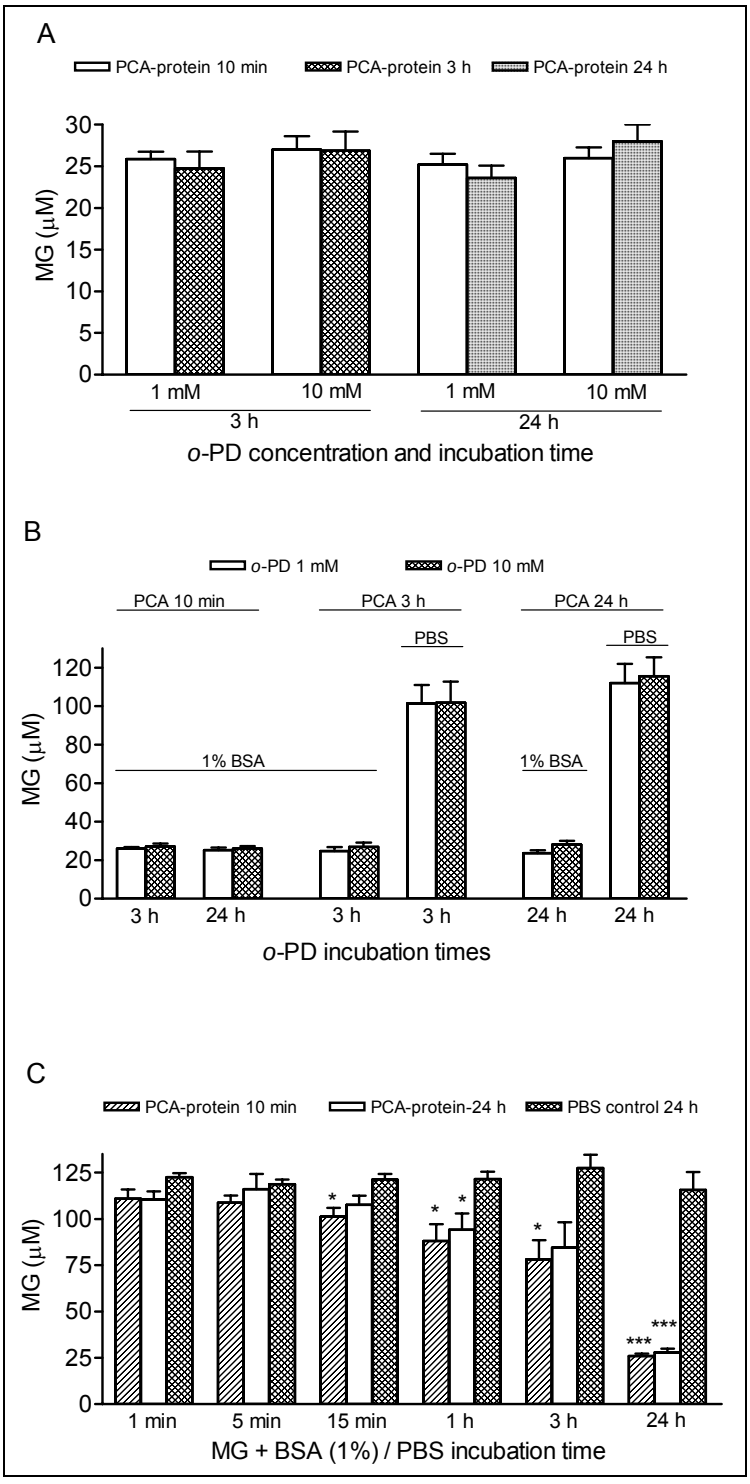

Fig. 5-5 Methylglyoxal (MG) levels in bovine serum albumin (BSA) samples measured with different protocols. MG (120 μM) was incubated with 1% BSA in 1 N PBS (MG-BSA) or 1 N PBS (MG-PBS) solution at 37° C for different times. The samples were then treated with

different protocols and subjected to HPLC for quantification of MG. A. The PCA (0.45 N)-precipitated protein was incubated in the sample (MG-BSA) for 10 min, 3 h or 24 h before removal by centrifugation. B. MG was derivatized by incubation with *o*-phenylenediamine (*o*-PD, 1 or 10 mM) for 3h or 24h. C. Decreasing MG levels after increasing incubation times with BSA. The PCA (0.45 N)-precipitated protein was incubated in the sample for 10 min, 3 h or 24 h. *o*-phenylenediamine (*o*-PD, 10 mM) was incubated for 24 h in all samples to derivatize MG. n = 3-4 for each group. *P<0.05, ***P<0.001 *vs*. corresponding PBS control.

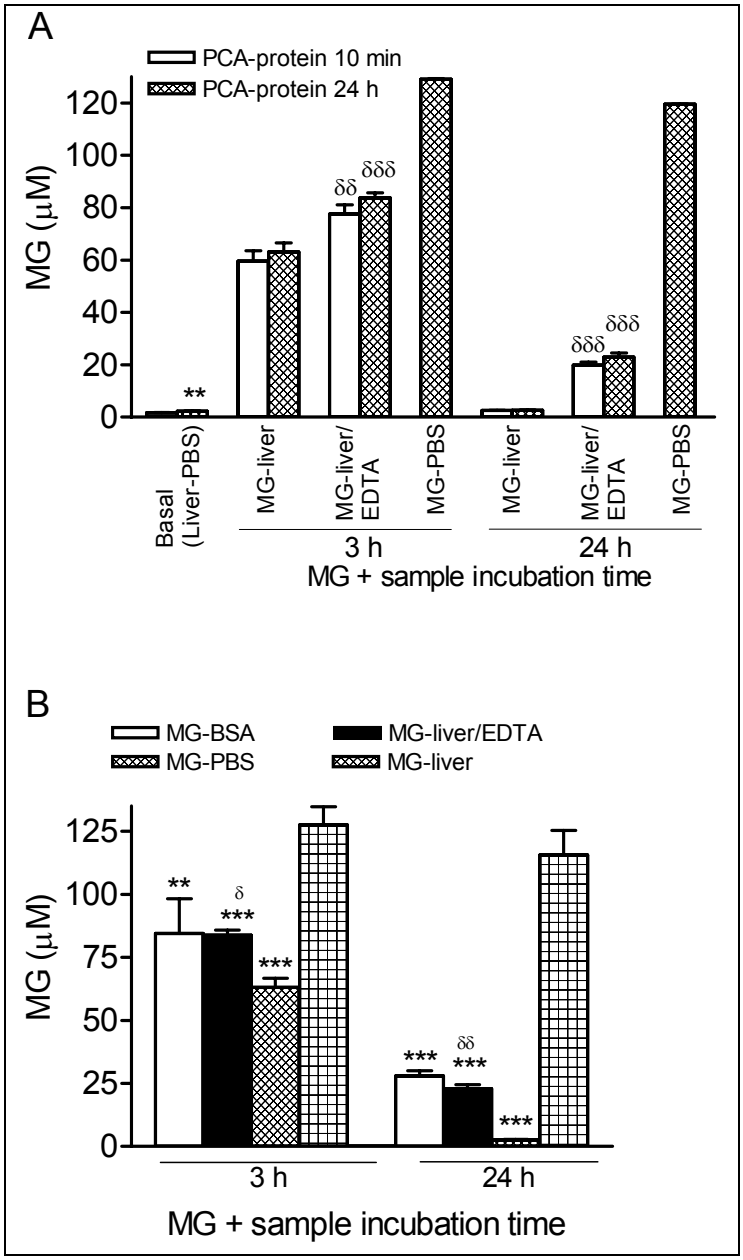

Fig. 5-6 Methylglyoxal (MG) levels in liver homogenate and bovine serum albumin (BSA) samples. Liver homogenate (see Methods) was dissolved in sodium phosphate (pH 4.5) to make a 1% solution and immediately assayed for MG (Basal). For other samples MG (120 μM) was incubated at 37° C for 3 or 24 h with 1% liver homogenate in 1 N PBS (pH 7.4) (MG-liver), 1% liver homogenate in sodium phosphate (pH 4.5) solution containing 50 μM

EDTA (MG-liver/EDTA), 1% BSA in 1 N PBS (pH 7.4) (MG-BSA) or 1 N PBS (MG-PBS). A. The 0.45 N PCA-precipitated protein was incubated in the sample for 10 min or 24 h before removal by centrifugation. MG was derivatized by incubation with *o*-phenylenediamine (10 mM) for 24 h. $^{**}P<0.01$ *vs*. corresponding PCA-protein 10 min value; $^{\delta\delta}P<0.01$, $^{\delta\delta\delta}P<0.001$ *vs*. corresponding MG-liver value. B. The 0.45 N PCA-precipitated protein along with *o*-PD (10 mM) was incubated in the sample for 24 h before removal by centrifugation. $^{**}P<0.01$, $^{***}P<0.001$ *vs*. corresponding MG-PBS value, $^{\delta}P<0.05$, $^{\delta\delta}P<0.01$ *vs*. corresponding MG-liver value.

4. Discussion

The consumption of excess carbohydrates, high blood glucose levels and the incidence of diabetes are increasing at an alarming rate in North America. High glucose is associated with elevated plasma MG levels. The high reactivity of MG with proteins and its implications are coming under increased scrutiny. Variations in the amount of MG reported are a source of confusion in the literature review involving MG. Chaplen *et al.* (Chaplen *et al*., 1998) reported that variations in the protocol for sample treatment and preparation for HPLC can yield significantly different values of MG from the same sample when tested on cultured Chinese hamster ovary cells. The effect of variations in protocol on the amount of MG detected in different biological samples has not been reported. Our results with variations in protocol when applied to commonly used biological samples are unexpected and very interesting.

Plasma samples are the ones most commonly analyzed and varying plasma concentrations have been reported in human (McLellan *et al*., 1992; Thornalley *et al*., 1989; McLellan *et al*., 1992; Beisswenger *et al*., 1999; Odani *et al*., 1999) as well as rat (Nagaraj *et al*., 2002; Wang *et al*., 2007). Plasma typically contains a mixture of numerous proteins without any cells. The bulk of the protein is albumin and globulin with lesser amounts of other proteins. Our results revealed significantly different values from the same plasma sample treated with different protocols in some instances. Use of 0.45 N PCA, as compared to 0.2 N, yielded higher values of MG in many instances (Fig. 5-2A). It is likely that a stronger acidic environment releases more MG from its reversible binding to proteins and other cellular components (Chaplen *et al*., 1998). Incubation of the PCA-precipitated protein for 24 h or even for 3 h did not yield significantly different values of MG as compared to 10 min of incubation except in a couple of instances (Fig. 5-2B). For example, there was a marked

difference in values when 10 mM of *o*-PD was used along with 0.45 N PCA incubated for 24 h as compared to PCA-protein for 10 min or 1 mM *o*-PD (Fig. 5-2B, 5-2C). A 10 mM concentration of *o*-PD did not give any interfering peak (Chaplen *et al*., 1998) on the HPLC when ran alone as a control. It should be noted that the difference was not as striking as with Chinese hamster ovary cells, which had more than 100 to 1000 fold difference in MG values (Chaplen *et al*., 1998). Also, we found that the plasma sample did not require passage through C_{18} solid-phase extraction (SPE) cartridge after precipitation with PCA (Randell *et al*., 2005). Our chromatograms were very clean within the regions of 2MQ and 5-MQ peaks (Fig. 5-1). Moreover, sample concentration was not necessary since the values detected were way above the detection limits. Moreover, passage through a column is likely to result in loss of sample and possibly some MG adducts. To begin with, plasma samples have limited volumes (200 – 400 µl) and loss in a column cannot be afforded. Thus, incubation of plasma samples with 0.45 N PCA-precipitated proteins and 10 mM *o*-PD for 24 h yielded very consistent values of MG in the plasma and is recommended.

Treatment of liver samples with 0.45 N PCA instead of 0.2 N gave consistently higher values of MG (Fig. 5-3A). Also, incubation of PCA-precipitated protein for 3 h or 24 h, as opposed to 10 min, resulted in higher values of MG in all instances (Fig. 5-3B). Our homogenized liver samples were reconstituted in an appropriate amount of sodium phosphate buffer (pH 4.5) and did not require sample concentration by passage through a C_{18} SPE column, as is necessary with cultured cells, especially when the dilute supernatant is analyzed for MG levels (Chaplen *et al*., 1998). We recommend incubation of liver and other organ samples with 0.45 N PCA-precipitated proteins and 10 mM *o*-PD for 24 h.

Analysis of cultured VSMCs showed significant differences in MG values when 0.45

N PCA was used instead of 0.2 N (Fig. 5-4A). A 24 h incubation with the 0.45 N PCA-precipitated protein gave consistently higher values of MG. Use of 10 mM *o*-PD as against 1 mM did not give impressive differences in MG values (Fig. 5-4C). We analyzed intracellular MG instead of MG in the culture medium. Accordingly the sample did not require concentration by passage through a SPE column. Since the culture medium was washed out there was no question of contamination with phenol red that is reported to give an interfering peak and requires removal by a SPE column (Chaplen *et al.*, 1998). For cultured cells we recommend incubation of samples with 0.45 N PCA-precipitated proteins and 1-10 mM *o*-PD for 24 h.

In order to determine more accurately the binding of MG to protein and the impact of different protocols on the amount measured we incubated a known amount of MG with 1% BSA for different times and subjected the same sample to different protocols (Fig. 5-5). Surprisingly incubation of the 0.45 N PCA-precipitated protein for 10 min, 3 h or 24 h did not affect the amount of MG detected. This implies that a longer incubation of the protein in acid either did not release more MG from its reversible binding or that the MG was not reversibly bound to BSA. However, Lo *et al* (Lo *et al*., 1994) have shown that when BSA was incubated with MG for up to 6 days almost half of it was irreversibly bound within 24 h to the arginine residues in BSA. About another quarter of the added MG was reversibly bound which remained reversibly bound over 6 days of incubation (Lo *et al*., 1994). Use of 1 or 10 mM *o*-PD also did not affect the amount of MG detected. Our results (Fig. 5-5C) also indicate that measurement of MG production in cultured cells is maximum in a time window of 1 min to 3 h after the cells start producing increased MG in response to a stimulus such as incubation with high glucose or fructose (25 mM or more) (Dhar *et al*., 2008). After 3 h a greater

proportion of MG binds to cellular components and becomes undetectable.

It is likely that the binding characteristics of MG are different with different proteins. Hence, we incubated MG with liver homogenate which contains an array of different proteins, lipids and other cellular components. Surprisingly, incubation of MG-liver samples with PCA for 10 min or 24 h did not affect the amount of MG detected (Fig. 5-6A). This implies that a longer incubation of the PCA-precipitated protein in acid either did not release more MG from its reversible binding or that the binding of MG to liver homogenate components is not reversible, the latter being highly unlikely. Moreover, after 24 h of incubation of MG with liver homogenate in PBS, in contrast to liver/EDTA, the amount of MG detected was not different from the basal (without any added MG) values in the same sample (Fig. 5-6A).

In contrast to detection of 66% and 24% MG after incubation of MG with 1% BSA for 3 h and 24 h respectively, we detected 49% and 2% MG after incubation of MG with 1% liver homogenate for 3 h and 24 h in 1 N PBS, respectively (Fig. 5-6B). The greatly reduced detection of MG from liver homogenate could be either due to metabolism by liver enzymes or due to increased irreversible binding of MG to liver proteins and other cellular components. To characterize this further, we prepared liver samples in sodium phosphate (pH 4.5) containing 50 μM EDTA with a view to minimize enzyme activity in the sample. Incubation of MG with this sample resulted in amounts of MG that were similar to MG-BSA sample indicating equivalent detectable fractions and similar protein binding characteristics of MG in both samples (Fig. 5-6B). It also indicated the possibility that the reduced MG in MG-liver sample was due to degradation of added MG by metabolic activity in the homogenate. Thus, addition of 50 μM EDTA to organ samples, though not necessary, is recommended.

Results with MG-BSA and MG-liver homogenate raise the question why more MG is

detected by different protocols from the same sample when applied to plasma, VSMC and especially liver samples. Plasma is mostly constituted of different proteins with hardly any cells hence, formation of MG from glycolytic intermediates such as dihydroxyacetone phosphate and DNA (Chaplen *et al*., 1998) can be safely ruled out. In the case of liver and VSMC samples formation of MG from glycolytic intermediates and DNA is possible. However, the strongly acidic environment created by 0.45 N PCA can be assumed to prevent formation of MG from glycolytic intermediates.

The detection of 100 to 1000 fold more MG from Chinese hamster ovary cells (Chaplen *et al*., 1998) after incubation of the sample with 0.45 N PCA-precipitated material for 24 h as opposed to 10 min is truly surprising as the authors describe it. One reason for this could be the excessively high glucose (100 mM) which they used for preincubation for 24 h. This can lead to very high amounts of MG formation that can possibly overwhelm the catalytic glyoxalase enzymes that efficiently remove MG. The glucose concentration in normal culture media is 5 mM (Chaplen *et al*., 1996). In one of our studies incubation of VSMCs with 25 mM glucose or fructose for 3 h resulted in between 3.5 and 4 fold increase in MG production along with a significant increase in oxidative stress when compared to untreated control cells (Dhar *et al*., 2008). It should be noted that plasma glucose levels are in the range of 20-25 mM in the severe diabetes seen in STZ-induced diabetic rats (Cheng *et al*., 2001).

MG values in lens tissue are also analyzed and perhaps routine analysis of adipose tissue or muscle tissue may become possible. Routine analysis of plasma MG in other groups of patients such as hypertensives or those on a ketogenic diet or Atkins diet which has high fat content, and in obese people, may soon become a reality (Dhar *et al*., 2008). Our results make a significant observation that the differences in MG levels in plasma, liver and VSMC samples

obtained with different protocols were most probably not due to protein binding characteristics of MG as indicated by the MG values in samples prepared by reacting MG with BSA and liver homogenate. In the latter samples no differences were observed in the amount of MG detected by varying the protocol, including a longer incubation of the PCA-precipitated protein in the sample with *o*-PD.

5. Conclusions

Variations in sample treatment protocol result in significant differences in the amount of MG detected in plasma, cultured VSMC and especially liver samples. Incubation of the sample with 0.45 N PCA and 10 mM *o*-PD for 24 h gave consistent values and is recommended for plasma, liver and other organ samples. Addition of 50 μM EDTA to organ samples reconstituted in sodium phosphate buffer (pH 4.5), though not necessary, is recommended. For cultured cells we recommend incubation of samples with 0.45 N PCA-precipitated proteins and 1-10 mM *o*-PD for 24 h. Our results may help in choosing a protocol that yields consistent values of MG in a given biological sample.

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CHAPTER 6

Alagebrium attenuates acute methylglyoxal induced glucose intolerance in Sprague-Dawley rats

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Abstract

Background and purpose: Alagebrium (ALA) is a novel advanced glycation endproducts (AGEs)-cross-link breaking compound. However, acute effects of ALA on major precursors of AGEs such as methylglyoxal (MG) have not been reported. MG is a highly reactive endogenous metabolite and its levels are elevated in diabetic patients. We investigated whether ALA attenuates the acute effects of exogenously administered MG on plasma MG levels, glucose tolerance and distribution of administered MG in different organs *in vivo* in Sprague-Dawley rats.

Experimental approach: We measured MG levels by HPLC, performed glucose tolerance test, adipose tissue glucose uptake, GLUT4, insulin receptor (IR) and insulin receptor substrate 1 (IRS-1) protein expression, and phosporylated IRS-1 in rats treated with MG at doses of either 17.25 mg/kg i.p. (MG-17 i.p.) or 50 mg/kg i.v. (MG-50 i.v.) with or without ALA, 100 mg/kg i.p.

Key results: ALA significantly attenuated the significant increases in MG levels in the plasma, aorta, heart, kidney, liver, lung, and urine after exogenous MG administration. In MG treated rats glucose tolerance was impaired, plasma insulin levels were higher and insulin-stimulated glucose uptake by adipose tissue was reduced than the respective control groups. In MG-50 i.v. treated rats GLUT4 protein expression and IRS-1 tyrosine phosphorylation were significantly reduced. ALA pretreatment attenuated these effects of MG. In an *in vitro* assay ALA significantly reduced the amount of detectable MG.

Conclusions and implications: Our results show for the first time that ALA acutely attenuates MG-induced glucose intolerance suggesting a possible preventive role for ALA against harmful MG effects.

Key words: Alagebrium, Methylglyoxal, glucose intolerance, diabetes.

Introduction

Alagebrium (4,5-dimethylthiazolium, ALA) (formerly known as ALT-711) (Fig. 1) is a novel advanced glycation endproducts (AGEs) cross-link breaking compound which has been studied mainly for its chronic effects on AGEs (Coughlan *et al*., 2007; Guo, Y *et al*., 2009; Little *et al*., 2005; Peppa *et al*., 2006; Susic *et al*., 2004; Thallas-Bonke *et al*., 2004; Wolffenbuttel *et al*., 1998; Ulrich and Zhang, 1997; Zieman *et al*., 2007). The first AGEs cross-link breaking compound discovered was phenacylthiazolium bromide (PTB) in 1996. PTB reacts with and cleaves covalent cross-links of AGEs-derived proteins. PTB degrades rapidly and hence a more stable derivative alagebrium was developed. ALA (210 mg/kg twice a day for 8 weeks) given to patients with systolic hypertension reduced vascular fibrosis and markers of inflammation (Zieman *et al*., 2007). Intraperitoneal injection of ALA (1 mg/kg) daily for 1 or 3 weeks reversed diabetes-induced increase of arterial stiffness measured by *in vivo* and *in vitro* parameters in STZ-induced diabetic rats, and improved impaired cardiovascular function in older rhesus monkeys (Wolffenbuttel *et al*., 1998; Ulrich and Zhang, 1997). ALA (10 mg/kg for 16 weeks) also increased glutathione peroxidase and superoxide dismutase activities in aging rats and reduced oxidative stress (Guo *et al*., 2009). However, it has not been shown if ALA has acute effects against precursors of AGEs such as methylglyoxal (MG) and glyoxal.

MG, a highly reactive dicarbonyl compound, is a metabolite of glucose, fatty acid, and protein metabolism (Desai and Wu, 2007; Thornalley, 1996). The clinical significance of MG lies in the fact that it reacts with and modifies certain proteins to form advanced glycation end

products (AGEs) (Desai and Wu, 2007; Thornalley, 1996; Vlassara, 2002). Among other things AGEs are implicated in the pathogenesis of vascular complications of diabetes (Vlassara, 2002). Plasma MG levels in healthy humans are 1 μM or less and are elevated to 2-6 μM in diabetic patients with a positive correlation to the degree of hyperglycemia (Wang *et al*., 2007; McLellan *et al*., 1994). Sprague-Dawley (SD) rats fed chronically with fructose develop insulin resistance (Hwang *et al*., 1987; Jia and Wu, 2007). We have shown that incubation of vascular smooth muscle cells with 25 mM glucose or fructose for 3 h increases MG production 3.5 or 3.9 fold, respectively, and increases oxidative stress (Dhar *et al*., 2008). MG modifies the structure of the insulin molecule *in vitro*, in a way that impairs insulin-mediated glucose uptake in adipocytes (Jia *et al*., 2006). In cultured 3T3-L1 adipocytes MG (20 μM) decreased insulin-induced insulin-receptor substrate-1 (IRS-1) tyrosine phosphorylation and phosphatidylinositol (PI) 3-kinase (PI3K) activity (Jia and Wu, 2007). Incubation of cultured L6 muscle cells with high concentrations of MG (2.5 mM) for 30 min impaired insulin signaling (Riboulet-Chavey *et al*., 2006) and a very high dose of MG (500 mg/kg i.p.) elevated plasma glucose level in cats, by releasing glucose from the liver *via* an adrenergic mechanism (Jerzykowski *et al*., 1975). Despite all of these cellular and molecular studies on MG and insulin signaling the *in vivo* effect of exogenous MG administration on glucose tolerance, especially in pathologically relevant plasma MG concentrations, is not known.

Numerous studies have been carried out to study the toxicity of high concentrations of MG *in vitro* (up to 20 mM) (Sheader *et al*., 2001) and *in vivo* (100 mg/kg to 1 g/kg, i.p. or i.v.) . Similar high concentrations of exogenous MG have been employed in most *in vivo* and *in vitro* studies, which raises concern of whether these studies bear physiological or pathological

relevance (Riboulet-Chavey *et al.*, 2006; Ghosh *et al.*, 2006; Kalapos, 1999; Cantero *et al.*, 2007; Golej *et al.*, 1998; Berlanga *et al.*, 2005). Under physiological conditions the highly efficient glyoxalase system degrades MG into D-lactate (Thornalley, 1996) and keeps plasma MG levels at around 1 μM or less (Wang *et al.*, 2007; McLellan *et al.*, 1994). The glyoxalase system consists of two enzymes, glyoxalase I and glyoxalase II that require catalytic amounts of reduced glutathione (GSH) for its activity (Thornalley, 1996).

In the present study we have we determined an appropriate dose and route for administration of exogenous MG that would result in pathologically relevant plasma concentrations of MG in experimental animals. We used this dose to investigate the tissue/organ distribution of exogenously administered MG in these animals and the effects of acute elevation of plasma MG levels on glucose tolerance and plasma insulin levels. In adipose tissue from MG treated rats glucose uptake, GLUT4, insulin receptor (IR), insulin receptor substrate-1(IRS-1) protein expression and IRS-1 tyrosine phosphorylation was studied. More importantly, we examined whether ALA can prevent or attenuate these effects of exogenously administered MG.

Methods

Animals

Male 11-week old Sprague-Dawley (SD) rats from Charles River Laboratories (Quebec, Canada) were used according to guidelines of the Canadian Council on Animal Care. After one week of acclimatization the rats were fasted overnight before the experiments.

In vitro incubation of alagebrium with methylglyoxal

MG (10 μM) was incubated with or without ALA (100 μM) for different times at 37° C. After the given incubation time, the sample was analyzed for MG by HPLC as described below.

Determination of an appropriate dose and route of administration of MG: Effects of pretreatment with ALA

In view of the inherent bioavailability barriers associated with the oral route, administering MG in drinking water or by gavage was not considered suitable for acute administration of a single dose to achieve consistent plasma levels. We chose the intraperitoneal (i.p.) and intravenous (i.v.) routes to get consistent plasma levels of MG. In order to achieve a pathologically relevant plasma concentration of 2-5 μM MG (Wang *et al.*, 2007; McLellan *et al.*, 1994; Wang *et al.*, 2008; Baynes and Thorpe, 1999), we calculated a dose based on an average blood volume of 6 ml per 100 g body weight (Lee and Blaufox, 1985) for a 300 g rat and assumed complete absorption from the i.p. injection site into the circulation. We administered 17.25 mg/kg (240 μmol/kg) by a single i.p. injection (described hereafter as MG-17 i.p.) or 6.48 mg/kg/h (90 μmol/kg/h) by i.v. infusion for 2 h (12.96 or about 13 mg/kg, MG-13 i.v.) with or without ALA (ALA -100 mg/kg i.p.). ALA was administered 15 min before the administration of MG (described hereafter as pretreatment).

The continuous i.v. infusion was chosen to deliver a constant low dose of MG in the circulation and compare its plasma levels with those resulting from the i.p. injection. In another group of rats, MG (50 mg/kg i.v., described hereafter as MG-50 i.v.) was given as a bolus injection in order to achieve higher plasma MG level. The rats were anesthetized with thiopental sodium (100 mg/kg i.p.). The trachea was cannulated to allow spontaneous

respiration and the left jugular vein and right carotid artery were also cannulated. Blood samples were collected at 5, 15, 30, 60 and 120 min into ethylene diamine tetra acetic acid (EDTA) containing tubes. Plasma MG levels were determined by HPLC.

In vivo distribution of MG after exogenous administration

In rats treated with saline (control), MG-17 i.p., or MG-17 i.p. + ALA, selected organs, tissues, and urine were collected 3 h after administration of tested compounds and frozen in liquid nitrogen. The organs and tissues were finely ground and homogenized in liquid nitrogen and reconstituted in sodium phosphate buffer (pH 4.5) and sonicated (30 s, three times). The samples were assayed for MG by HPLC, as described below, and for protein measurement.

Intravenous Glucose Tolerance Test (IVGTT)

After overnight fasting, an intravenous glucose tolerance test (IVGTT) was performed as described previously (Laight *et al.*, 1999). Briefly, the trachea, left jugular vein, and right carotid artery were cannulated in anesthetized rats. After collecting a basal blood sample rats were treated with saline, MG or MG+ALA. After 2 h a 0 min blood sample was taken and a bolus dose of glucose (0.5 g/kg) was given i.v. and further blood samples were collected at 1, 3, 6, 12 and 24 min from the carotid artery. Plasma glucose levels were determined using a glucose assay kit (BioAssay Systems, Hayward, CA, USA) and insulin levels were measured with a rat insulin assay kit (Mercodia Rat Insulin ELISA). The IVGTT result was calculated as the area under the curve (AUC) for both plasma glucose and insulin levels between time 0 min and 24 min and expressed as arbitrary units.

Glucose uptake

Insulin sensitivity of adipose tissue was evaluated by measuring insulin-induced 2-Deoxy-[^{3}H] glucose (2-DOG) uptake as described previously (Jia and Wu, 2007). Briefly, abdominal visceral adipose tissue was chopped and digested in DMEM base (no glucose, no serum) with collagenase (1.5mg/ml) at 37° for 20 min. The mixture was filtered, centrifuged, supernatant discarded and the pellet was re-suspended in the same DMEM. Thereafter, the cells were exposed to 100 nM insulin for 30 min and continuously incubated for another 20 min after the addition of [^{3}H]-2-DOG (0.1 μCi/500μl) with glucose (50 μM) to the medium. The incubation was stopped by washing cells three times with ice-cold glucose-free phosphate buffer. The cells were lysed in 0.1% sodium dodecyl sulfate (SDS) and 1 N NaOH and transferred into scintillation vials for counting (Beckman LS 3801 scintillation counter).

Preparation of total membrane fraction from adipose tissue for GLUT4

Abdominal visceral adipose tissue isolated from rats was homogenized in buffer B [10 mmol/l Tris–HCl, 1 mmol/l EDTA, 250 mmol/l sucrose and 0.1 mmol/l phenylmethylsulfonyl fluoride (PMSF, pH 7.4)] using a polytron homogenizer. The homogenate was centrifuged at 1700×*g* for 10 min at 4° C and the resulting supernatant was centrifuged at 8600 x *g* for 10 min at 4° C. The supernatant was then centrifuged at 185000 x *g* for 60 min at 4 °C, and stored at −70° C before use (Furuta *et al.*, 2002). The protein concentration of the supernatant was determined by the bicinchoninic acid (BCA) protein assay reagent.

Immunoprecipitation and western blotting

For immunoprecipitation abdominal visceral adipose tissue was lysed in an ice-cold

radioimmunoprecipitation assay buffer (RIPA) buffer containing 30 mM Hepes (pH 7.4), 5 mM EDTA; 1% Nonidet P-40, 1% Triton X-100, 0.5% sodium deoxycholate, 8 mM Na_3VO_4, 1 mM NaF and 2 mM protease inhibitor (Jia and Wu, 2007). Tissue homogenates were incubated with IRS-1 antibody for two hours at 4º C, followed by incubation with Protein A/G-Agarose for further two hours at 4º C. Immunoprecipitates were separated using spin-collection filters and washed once with RIPA buffer and three times with PBS. For western blotting, cell lysates or membrane fractions (50 µg) were boiled with sample buffer for 5 min, resolved by 10–12% SDS-PAGE, and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). The membranes were blocked and incubated with the anti-IR (Santa Cruz, CA, USA), anti-GLUT4 (Santa Cruz, CA, USA), and anti-*β*-actin antibodies (Santa Cruz, CA, USA), respectively, followed by incubation with horse radish peroxidase conjugated secondary antibodies (Upstate, MA, USA). The proteins were then visualized with chemiluminescence reagents (Amersham Biosciences, NJ, USA) and exposed to X-ray film (Kodak Scientific Imaging film, X-Omat Blue XB-1).

Methlyglyoxal assay

MG was measured by a specific and sensitive HPLC method as described previously (Dhar *et al.*, 2008) with some modifications to the original protocol (Chaplen *et al.*, 1998). MG was derivatized with *o*-phenylenediamine (*o*-PD) to specifically form 2-methylquinoxaline. The samples were incubated in the dark for 24 h with 0.45 N perchloric acid (PCA) and 10 mM *o*-PD at room temperature. Samples were centrifuged at 12000 rpm for 10 min. 2-methylquinoxaline and quinoxaline internal standard (5-methylquinoxaline) were quantified on a Hitachi D-7000 HPLC system (Hitachi, Ltd., Mississauga, ON, Canada)

via Nova-Pak® C18 column (3.9×150 mm, and 4 μm particle diameter, MA, USA).

Glutathione and D-lactate assays

GSH was measured by HPLC whereas D-lactate was measured by an assay kit ( ).

Chemicals and Statistical analysis

All chemicals were of analytical grade. Methylglyoxal and *o*-phenylenediamine (*o*-PD) were purchased from Sigma Aldrich, Oakville, ON, Canada. Alagebrium (formerly known as ALT-711) was a generous gift from Synvista Therapeutics, Inc. (Montvale, NJ, USA). Data are expressed as mean ± SEM and analyzed using one way ANOVA and *post hoc* Dunnett's test. *P* value less than 0.05 was considered significant. Data on tissue distribution of MG (Fig. 3) were analyzed with two-way ANOVA with treatment and tissue as two variables.

Results

Incubation of ALA with MG in vitro.

Incubation of MG (10 μM) with ALA (100 μM) for different times resulted in a significant reduction in the amount of MG detected by HPLC with increasing time of incubation. Even after 15 min of incubation the amount of MG detected was significantly reduced suggesting an acute effect of ALA (Table 6-1).

ALA attenuates increase in plasma MG levels following exogenous MG administration

After acute administration of MG-17 i.p. the plasma level of MG peaked at 15 min to reach 2.5 μM, 2.6 fold higher than the basal value (Fig. 6-2A). The MG level declined to a

plateau after 1 h but was still higher (2.1 fold) than the basal value even after 2 h. In another group of rats, after i.v. infusion of MG (6.48 mg/kg/h or 90 μmol/kg/h for 2 h) the plasma MG level peaked at 5 min to reach 2.7 μM, 2.7 fold higher than the basal value (Fig. 6-2B). Similarly, the plasma level of MG in this group declined gradually and was still significantly (2.2 fold) higher than the basal value after 2 h. Pretreatment with ALA (100 mg/kg) significantly prevented the increase in plasma MG after both i.p. and i.v. administration of MG (Fig. 6-2A and B), which most probably could be due to scavenging of MG by ALA. Thus, both routes of administration (i.p. & i.v.) can increase plasma MG levels in similar pattern to a level comparable to that under various pathological conditions reported in the literature (Wang *et al.*, 2007; McLellan *et al.*, 1994; Wang *et al.*, 2008; Baynes and Thorpe, 1999). Therefore, MG-17 i.p. was chosen for most of the following studies. Administration of a higher dose of MG-50 i.v. resulted in significantly higher plasma MG levels than with MG-17 i.p. or MG-13 i.v. and ALA attenuated the increase in plasma MG (Fig. 6-2C). MG-50 i.v. was administered to some groups of rats to assess dose-related severity of effects of MG on glucose tolerance and plasma insulin levels.

ALA attenuates distribution of MG in rats after exogenous administration

Following administration of MG-17 i.p. MG levels increased significantly in the aorta (1.6 fold), heart (1.4 fold), liver (1.3 fold), lungs (1.3 fold), and kidney (1.2 fold) compared to the basal levels in the control group (Fig. 6-3). The aorta had the greatest increase in the level of MG (11.2 ± 0.7 nmol/mg protein) compared to control (7.2 ± 0.3 nmol/mg protein) and had the highest levels amongst the organs or tissues tested (Fig. 6-3). Urinary MG level was also significantly higher (2.5 fold) in the MG-17 i.p. group compared to the control group (Fig. 6-

3). The increased MG levels in rats treated with MG-17 i.p. were significantly attenuated by pretreatment with ALA (Fig. 6-3). The urinary MG levels (Mean ± SEM, μM) in the MG-50 i.v. group were as follows: Control (saline), 25 ± 4 (n = 9); MG-50 i.v., 232 ± 45*** (n = 6); MG-50 i.v. + ALA, 134 ± 41** (n = 4); **P<0.01, ***P<0.001 *vs*. control group. There was no significant increase in MG levels in spleen and brain of rats after MG-17 i.p. administration. Inter-tissue variation in MG levels before and after MG or MG+ALA administration was significantly different as analyzed by two-way ANOVA (Fig. 6-3).

Impairment of glucose tolerance and glucose uptake in MG-treated rats is prevented by ALA

After acute MG-17 i.p. and MG-50 i.v. administration plasma glucose and AUC were determined in these rats (Fig. 6-4). MG-17 i.p. significantly impaired glucose tolerance with increased AUC, which was attenuated by pretreatment with ALA (Fig. 6-4A, B). The impairment of glucose tolerance was significantly greater in the MG-50 i.v. treated group than its control group (Figs. 6-4C, D). Pretreatment with ALA significantly attenuated impairment of glucose tolerance by MG and reduced the AUC (Fig. 6-4D).

Insulin-stimulated glucose uptake was evaluated in abdominal visceral adipose tissue freshly isolated from rats 2 h after administration of MG-17 i.p. or MG-50 i.v. or saline (control) in separate groups of rats. There was a significant decrease in insulin-stimulated glucose uptake in MG-17 i.p. treated rats and it was more severe in MG-50 i.v. treated rats compared to control. The reduced glucose uptake by both doses was prevented by pretreatment with ALA (Fig. 6-5).

Increased plasma insulin levels in MG-treated rats are attenuated by ALA

The basal plasma insulin levels were not different among the control, MG-treated and MG+ALA groups. Following an IVGTT, the plasma insulin levels were higher in rats treated with MG-17 i.p. and MG-50 i.v. (Fig. 6-6A, C). The AUC for plasma insulin levels after the IVGTT was significantly greater in MG-17 i.p. and MG-50 i.v. treated rats compared to respective control (Fig. 6-6B, D). Pretreatment with ALA significantly attenuated the increase in plasma insulin levels and AUC values induced by MG-17 i.p. (Fig. 6-6A, B).

ALA prevents decreased plasma GSH levels in MG-treated rats.

Rats treated with MG-17 i.p. had significantly reduced plasma GSH levels compared to the control rats (Table 6-2). Co-administration of ALA (100 mg/kg i.p.) with MG-17 i.p. significantly reversed the decrease in plasma GSH induced by MG-17 i.p. (Table 6-2).

Effects of MG and ALA on plasma and aortic D-lactate levels

D-lactate is a metabolite of MG (Desai and Wu, 2007). Plasma D-lactate levels were significantly elevated after MG-50 i.v. and even further elevated after MG-17 i.p.+ALA and MG-50 i.v.+ALA (Table 6-3). Aortic D-lactate levels (μmol/mg protein, n=3 each group) were also significantly elevated after MG-17 i.p. (7.7 ± 0.8*) and further elevated after MG-17 i.p.+ALA (9.6 ± 1.0**) compared to the control group (4.0±0.6) (*P<0.05, **P<0.01 *vs.* control group).

Effects of MG on insulin signaling pathway in adipose tissue are attenuated by ALA

In order to confirm the possible mechanism of MG induced glucose intolerance and reduced glucose uptake, the protein expression of GLUT4 (Fig. 6-7), IR, IRS-1 (Fig. 6-8) and tyrosine

phophorylation of IRS-1 (Fig. 6-9) was examined in MG-50 i.v. treated rats. There was significant decrease in GLUT4 protein expression in abdominal visceral adipose tissue from MG-50 i.v. treated rats compared to that from control rats (Fig. 6-7). There was no change in the protein expression of IR and IRS-1 (Fig. 6-8). However, insulin-induced tyrosine phosphorylation of IRS-1 was significantly reduced in MG-50 i.v. treated rats that was attenuated by pretreatment with ALA (100 mg/kg i.p.) (Fig. 6-9).

Table 6-1

ALA reduces detectable methylglyoxal. MG was incubated with ALA at 37° C for different times. The solution was analyzed for MG by HPLC after the given incubation period. The values are mean ± SEM (n = 4 each). *P<0.05, **P<0.01 *vs*. MG alone.

Time of *in vitro* incubation	**MG (10µM) alone**	**MG (10µM) + ALA (100 µM)**	**ALA (100 µM) alone**
	Amount of MG detected by HPLC		
15 min	**9.5 ± 0.7**	**6.9 ± 0.1***	**0**
30 min	**9.4 ± 0.7**	**6.8 ± 0.3***	**0**
1 h	**9.3 ± 0.7**	**5.8 ± 0.1****	**0**
2h	**9.4 ± 0.7**	**5.2 ± 0.9***	**0**
24 h	**9.4 ± 0.7**	**5.0 ± 0.1****	**0**

Table 6-2

Effect of saline (control), MG-17 i.p. (MG, 17.28 mg/kg intraperitoneally) and MG+ALA (alagebrium 100 mg/kg, i.p.) on plasma reduced glutathione (GSH) in Sprague-Dawley rats. The values are mean ± SEM (n = 6 each). ***P<0.01 *vs*. control group, $^{\delta}P$<0.05 *vs*. MG group.

	Control	**MG**	**MG+ALA**
Plasma GSH (µM)	**111 ± 5**	**35 ± 1*****	**61 ± 5*** δ**

Table 6-3

Effect of saline (control), MG-17 i.p. (MG, 17.28 mg/kg intraperitoneally), MG-50 i.v. (MG, 50 mg/kg intravenous) and MG+ALA (alagebrium 100 mg/kg, i.p.) on plasma D-lactate levels in Sprague-Dawley rats. The values are mean ± SEM (n = 4 each). *P<0.05, ***P<0.01 *vs*. control group.

	Treatment group				
	Control	**MG-17 i.p.**	**MG-17 i.p. + ALA**	**MG-50 i.v.**	**MG-50 i.v. + ALA**
Plasma D-lactate (mM)	4.6 ± 0.3	6.0 ± 0.9	7.8 ± 1.0*	7.5 ± 1.0*	8.9 ± 0.5***

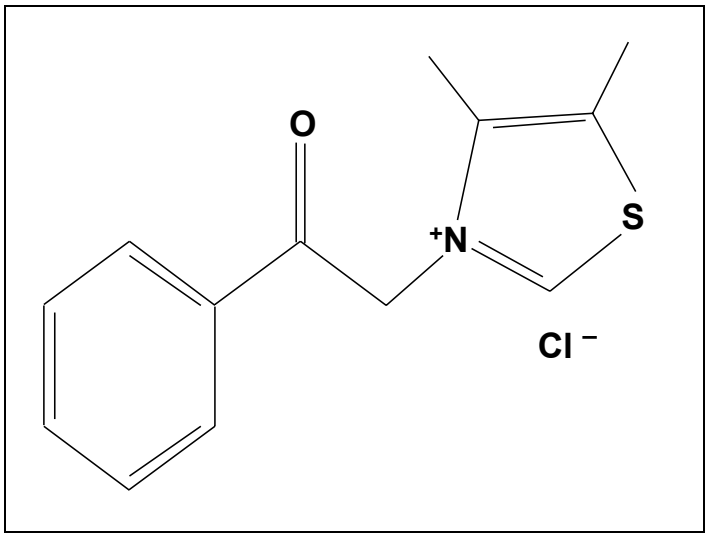

Fig. 6-1 Chemical Structure of alagebrium (4,5-dimethylthiazolium)

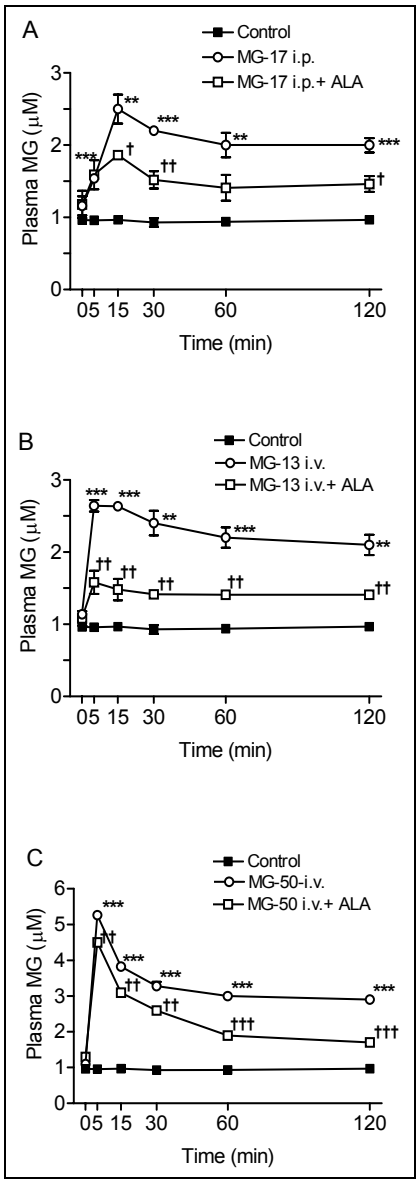

Figure 6-2 Plasma methylglyoxal (MG) levels after (A) intraperitoneal (i.p.) or (B, C) intravenous (i.v.) administration of MG in SD rats. n = 6 for each group. Control: saline injection; MG-17 i.p.: MG 17.25 mg/kg i.p.; MG-13 i.v.: MG 6.48 mg/kg/h i.v. infusion for 2 h; MG-50 i.v.: MG 50 mg/kg i.v. slow bolus injection; ALA: algebrium 100 mg/kg i.p. was given 15 min before the administration of MG in A, B and C. $^{**}P<0.01$, $^{***}P<0.001$ compared to control at same time point, $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ compared to respective MG-17 i.p., MG-13 i.v. or MG-50 i.v. treated group at the same time point.

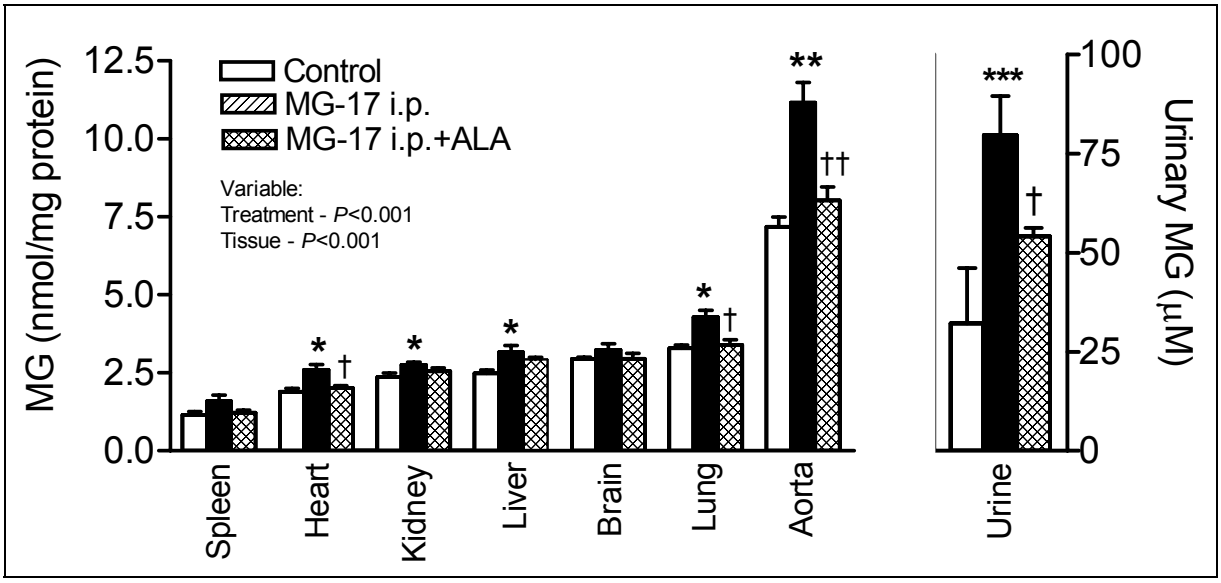

Figure 6-3 Distribution of methylglyoxal (MG) in different organs/tissues/urine in Sprague-Dawley rats after intraperitoneal administration. Saline (control), MG (17.25 mg/kg i.p., MG-17 i.p.) or MG-17 i.p. + ALA (alagebrium 100 mg/kg i.p.) were administered to three groups of rats (n = 6 each). The organs, tissues and urine were collected 3 h after administration of treatment. Data were analyzed with two-way ANOVA with treatment and tissue as variables. *P<0.05, **P<0.01, ***P<0.001 *vs*. corresponding control group, $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01 *vs*. MG-17 i.p. group.

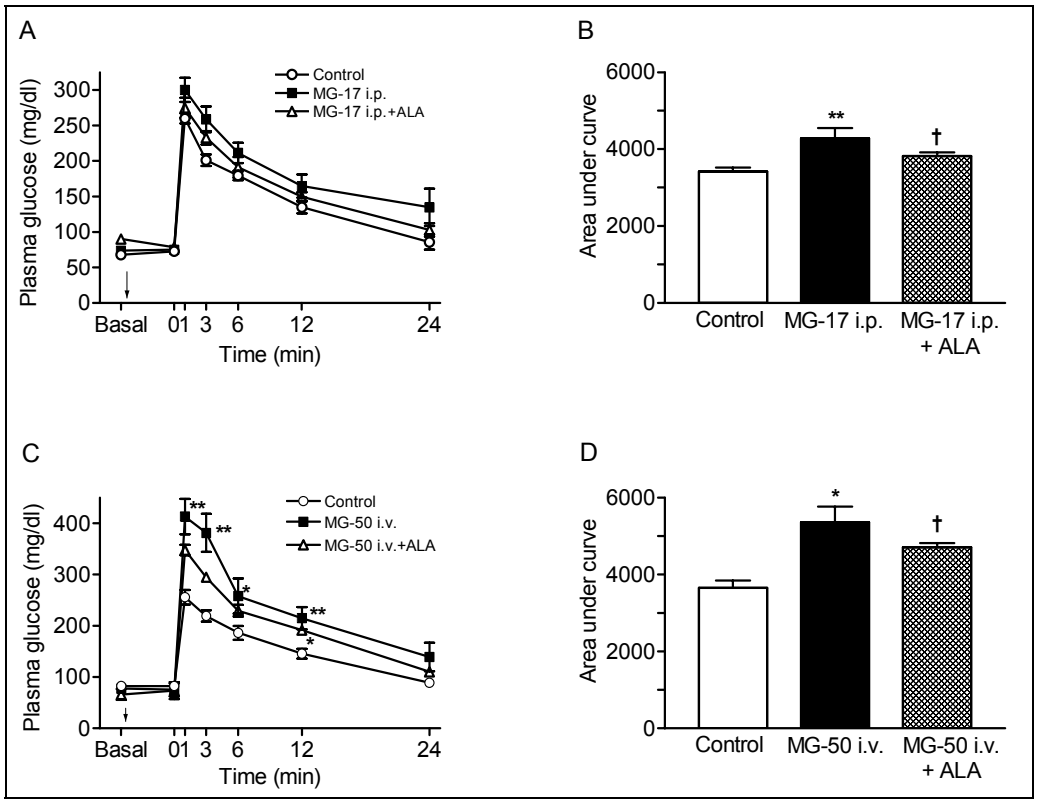

Figure 6-4 Intravenous glucose tolerance test (IVGTT) in MG-treated Sprague-Dawley rats, effect of ALA. Basal plasma glucose levels were determined before any treatment. The plasma glucose levels (A) and area under curve (B) were evaluated in rats for 24 min during an IVGTT which was performed 2 h after treatment with saline (control), MG-17 i.p. or MG-17 i.p. + ALA (alagebrium 100 mg/kg i.p.). The plasma glucose levels (C), area under curve (D) were evaluated in rats for 24 min during an IVGTT which was performed 2 h after treatment with saline (control), MG-50 i.v. or MG-50 i.v + ALA (alagebrium 100 mg/kg i.p.). 2 h after saline or drugs a time 0 plasma sample was obtained before giving a glucose load (0.5 g/kg i.v.) to perform the IVGTT. (C) **P*<0.05, ***P*<0.01 compared to control group at the same time point, (B, D) **P*<0.05, ***P*<0.01 compared to respective control group, $^{\dagger}P$<0.05 compared to respective MG treated group**.** n = 9 in each group.

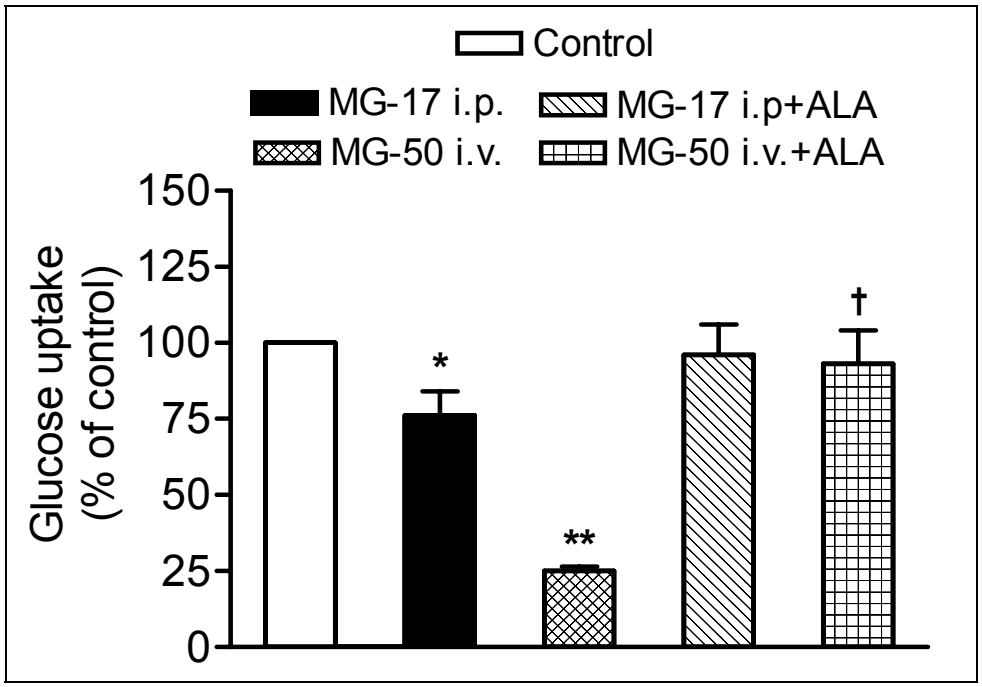

Figure 6-5 Adipose tissue glucose uptake in MG-treated Sprague-Dawley rats. Glucose uptake by adipose tissue was evaluated in five groups of rats 2 h after treatment with saline (control); MG 17.25 mg/kg i.p. (MG-17 i.p.); MG-17 i.p.+ALA (alagebrium 100 mg/kg i.p. given 15 min before the administration of MG); MG 50 mg/kg i.v. slow bolus injection (MG-50 i.v.) and MG-50 i.v.+ALA (alagebrium 100 mg/kg i.p.). Visceral adipose tissue was removed from the abdomen and tested for insulin-stimulated glucose uptake *in vitro*. *P<0.05, **P<0.01 compared to control, $^{\dagger}P$<0.05 compared to MG-50 i.v. treated group. n = 4 for each group.

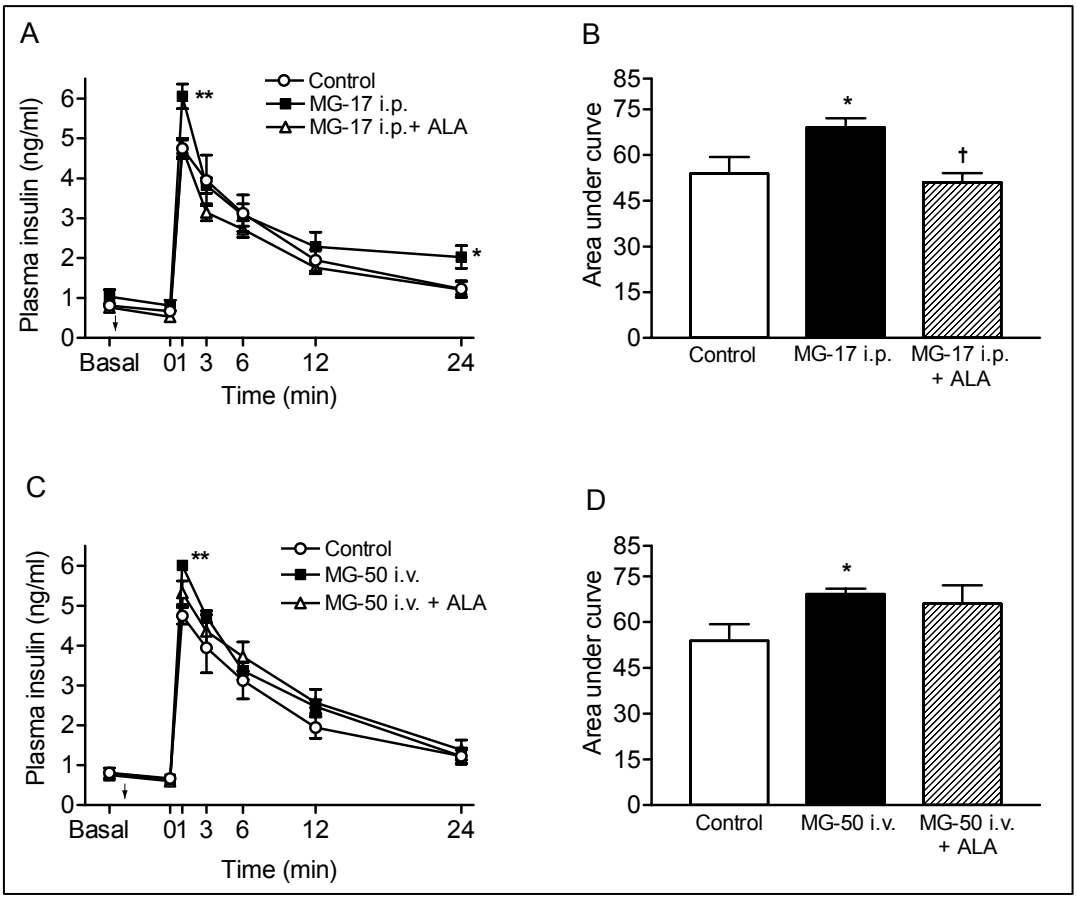

Figure 6-6 Plasma insulin levels in MG-treated Sprague-Dawley rats, effect of ALA. Basal plasma insulin levels were determined before any treatment. The plasma insulin levels (A) and area under curve (B) were evaluated in the rats for 24 min during an IVGTT which was performed 2 h after treatment with saline (control), MG-17 i.p., or MG-17 i.p. + ALA (alagebrium 100 mg/kg i.p.). The plasma insulin levels (C) and area under curve (D) were evaluated in the rats for 24 min during an IVGTT which was performed 2 h after treatment with saline (control), MG-50 i.v., or MG-50 i.v. + ALA (alagebrium 100 mg/kg i.p.). 2 h after saline or drugs a time 0 plasma sample was obtained before giving a glucose load (0.5 g/kg i.v.) to perform the IVGTT. *P<0.05, **P<0.01 compared to respective control group, $^{\dagger}P$<0.05 compared to MG-17 i.p. group. n = 9 for each group.

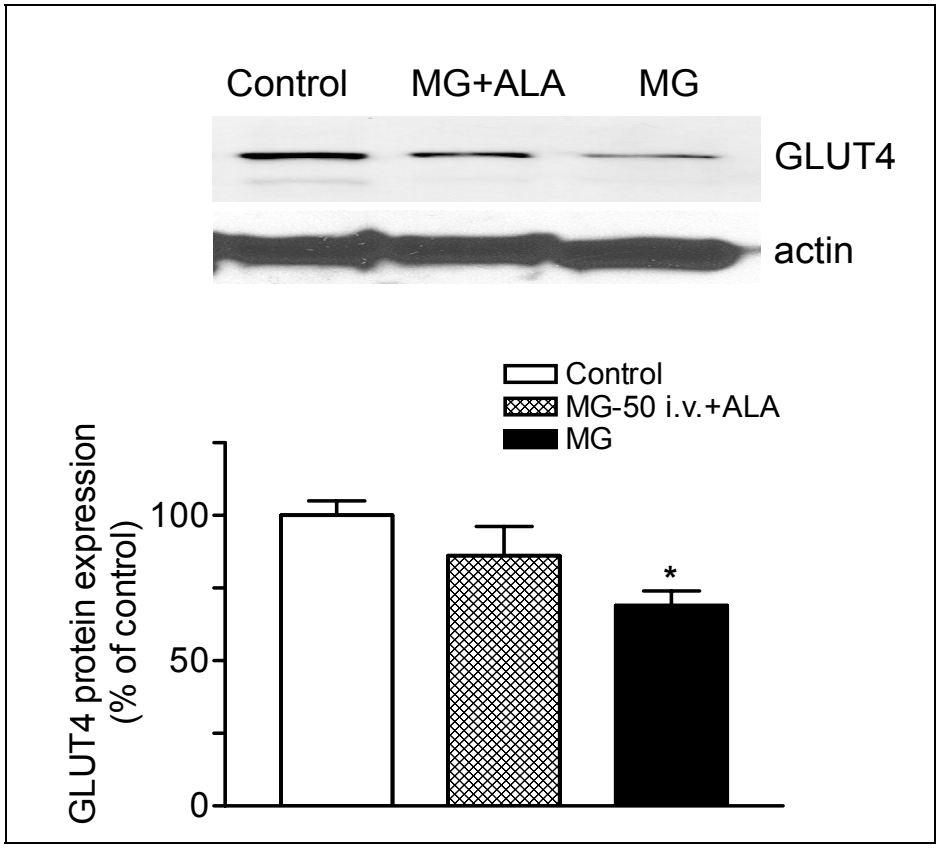

Figure 6-7 GLUT4 protein expression in MG treated rats. Groups of rats were treated with saline (Control), MG-50 i.v. or MG-50 i.v. + ALA (alagebrium 100 mg/kg i.p.). After 2 h the abdominal adipose tissue was removed and processed for determination of GLUT4 protein expression by western blotting. **P*<0.05 compared to control. *n* = 4 for each group.

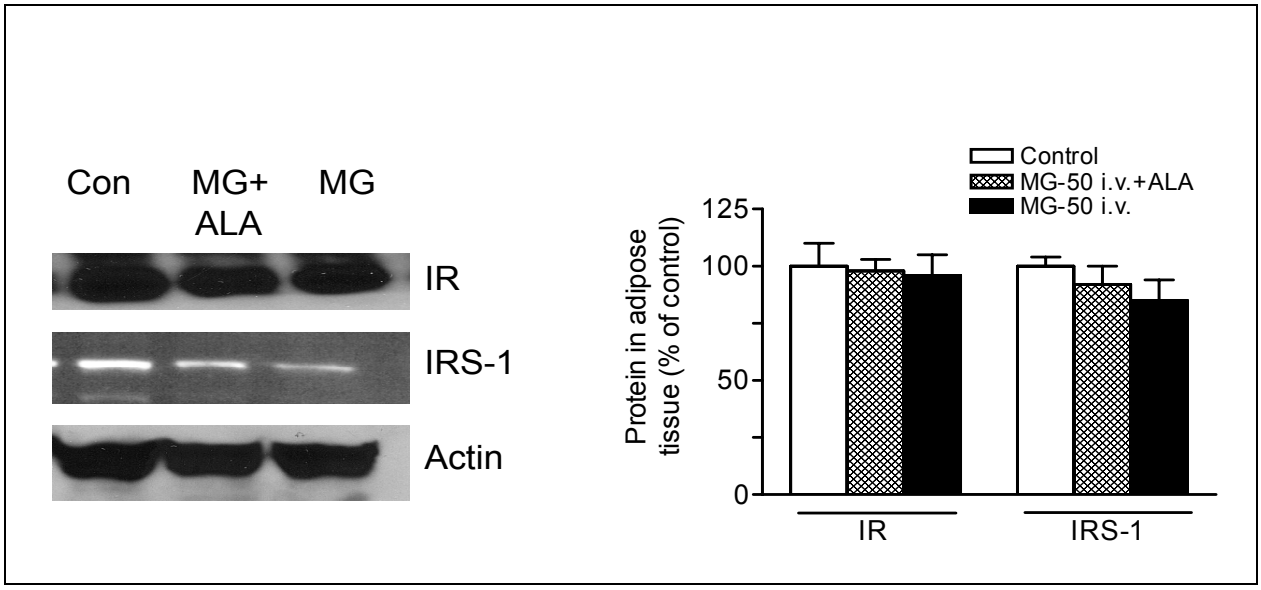

Figure 6-8 Insulin receptor (IR) and insulin receptor substrate 1 (IRS-1) protein expression in MG treated rats. Groups of rats were treated with saline (Control), MG-50 i.v. or MG-50 i.v. + ALA (alagebrium 100 mg/kg i.p.). After 2 h the abdominal adipose tissue was removed and processed for determination of IR and IRS-1 protein expression by western blotting. $n = 4$ for each group.

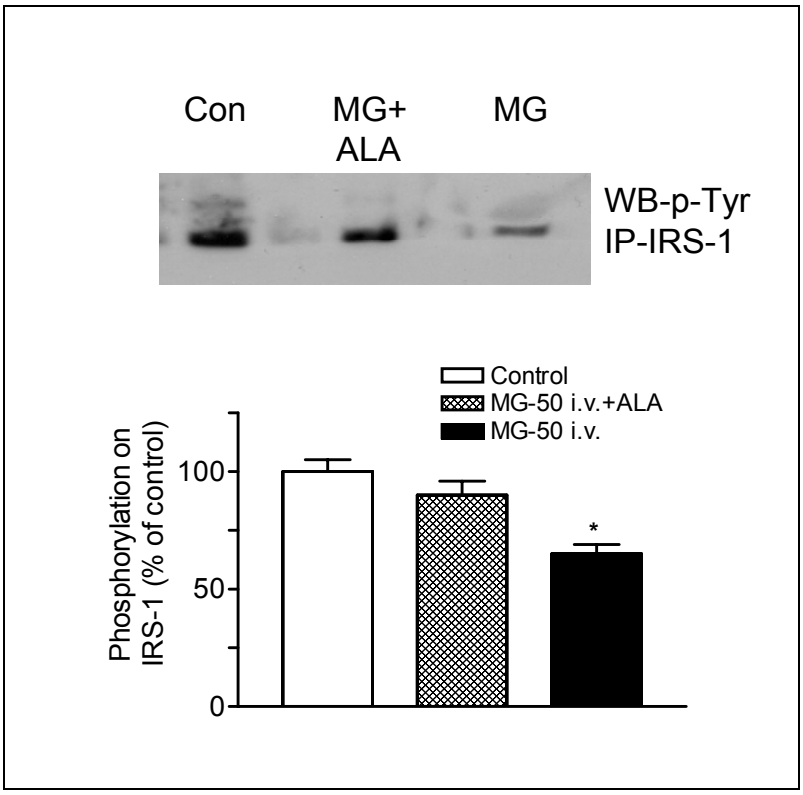

Figure 6-9 Insulin receptor substrate 1 (IRS-1) tyrosine phosphorylation in MG treated rats. Groups of rats were treated with saline (Control), MG-50 i.v. or MG-50 i.v. + ALA (alagebrium 100 mg/kg i.p.). After 2 h the abdominal adipose tissue was removed and tissue lysates were subjected to immunoprecipitation (IP) with IRS-1 antibody. The immunoprecipitates were then subjected to Western blotting (WB) using anti-pTyr. The immunoreactivity level was compared to the control level of IRS-1 phosphorylation. *$P < 0.05$ *vs.* control rats, $n = 4$ for each group.

Discussion

In the present study we report for the first time that ALA has acute preventive effects against the harmful of effects of the AGEs precursor MG *in vivo*. ALA is a well documented AGEs cross-link breaking compound in chronic studies (Coughlan *et al*., 2007; Guo, Y *et al*., 2009; Little *et al*., 2005; Peppa *et al*., 2006; Susic *et al*., 2004; Thallas-Bonke *et al*., 2004; Wolffenbuttel *et al*., 1998; Ulrich and Zhang, 1997; Zieman *et al*., 2007). We also show for the first time that acute administration of a single dose of MG adversely affects glucose tolerance in SD rats. When MG was administered in a lower dose (17.25 mg/kg, i.p.: MG-17 i.p.) the plasma MG levels were elevated to the pathologically relevant concentrations observed in diabetic patients (Wang *et al*., 2007; McLellan *et al*., 1994), for more than 2 hours. With this acute elevation of circulating MG, glucose tolerance of the rats was impaired; glucose-stimulated plasma insulin level increased, insulin-stimulated glucose uptake in the adipose tissue was reduced and urinary MG levels and aortic tissue content of MG increased. To achieve a higher plasma MG level it was administered at a higher dose (50 mg/kg i.v.: MG-50 i.v.) in separate groups of rats. Indeed with MG-50 i.v., an even higher plasma MG level (Fig. 6-2B) was obtained that also significantly impaired glucose tolerance, increased plasma insulin levels, reduced insulin-stimulated glucose uptake in adipose tissue along with a significant reduction in GLUT4 protein expression and tyrosine phosphorylation of IRS-1. ALA, administered i.p. 15 min prior to MG, attenuated all of these acute effects of MG and the increase in plasma levels following MG administration. *In vitro* incubation of ALA with MG for different times, starting with 15 min, significantly reduced the amount of MG detected in the sample (Table 6-1) possibly suggesting binding (scavenging) of MG by ALA. The attenuation by ALA of increased plasma MG levels following exogenous MG administration

also suggests a scavenging or binding effect of ALA on MG. To the best of our knowledge an acute scavenging or binding effect of ALA on MG has not been reported before. Phenacylthiazolium bromide (PTB) was the first AGEs cross-link breaking compound reported in 1996 but it degrades rapidly (Vasan *et al*., 1996). ALA is a more stable thiazolium derivative (Fig. 6-1) (Desai & Wu, 2007) and was developed based on an earlier observation that the carbon-carbon bond of α-diketones can be selectively cleaved with some thiazolium salts (Vasan *et al*., 1996). Thus, our results show that ALA has additional acute upstream effects that can prevent AGEs formation from MG, which can be useful for preventive treatment of AGEs related disorders.

The *in vivo* fate of exogenously administered MG is unknown. Our results show for the first time that the majority of administered MG-17 i.p. is excreted in the urine, an effect attenuated by ALA (Fig. 6-3). Since proteins are not filtered from the glomerular capillaries, the presence of MG in the urine indicates that most of the administered MG is likely in free form in the plasma, at least initially, and gets filtered into the urine. When ALA is present the free MG likely binds to ALA and urinary excretion of MG is reduced. We have observed that when MG is incubated with bovine serum albumin at 37º C, more than 90% is free, i.e. not protein bound, up to the first 15 min of incubation (Dhar *et al*, 2009).

After MG-17 i.p. the aortic MG increased significantly more compared with the other six organs investigated, including the heart and lungs. This increase in MG level was attenuated by ALA. The high basal as well as post-MG administration levels of aortic MG are of great pathological significance in terms of development of MG-induced AGEs and atherogenesis, and endothelial dysfunction over a long term (Thornalley, 1996; Desai and Wu, 2007; Vlassara and Palace, 2002). There was no significant increase in MG levels in the

spleen and the brain as compared to control. The reason for the increased basal as well as post-administration MG in the aorta and the uneven organ distribution needs further separate studies.

The plasma levels of MG are around 1 μM in normal SD rats (Fig. 6-2) (Wang *et al.*, 2008) and 1 μM or less in healthy humans (Wang *et al.*, 2007; McLellan *et al.*, 1994). Under physiological conditions, the glyoxalase system rapidly degrades MG into D-lactate which minimizes its reaction with proteins and other cellular components to form AGEs. GSH is an essential component of the glyoxalase system. (Thornalley, 1996; Baynes and Thorpe, 1999; Desai and Wu, 2007). We found reduced GSH levels in rats treated with MG-17 i.p. (Table 6-2). Also, in hyperglycemia and diabetic patients the plasma MG levels are elevated to between 2 and 6 μM (Wang *et al.*, 2007; McLellan *et al.*, 1994) with associated oxidative stress and reduced GSH levels (Baynes and Thorpe, 1999). The enzymes glutathione reductase and glutathione peroxidase play a key role in the recycling of glutathione between its reduced (GSH) and oxidized (GSSG) forms. Glutahione peroxidase removes hydrogen peroxide with the help of GSH that is in turn oxidized to GSSG. Glutathione reductase acts as an antioxidant by converting GSSG to GSH. MG can increase oxidative stress by causing glycation of glutathione reductase and glutathione peroxidase and inactivating them (Desai and Wu, 2008). MG has also been shown to directly deplete GSH in various cell types so that the cell becomes more sensitive to oxidative stress. Reduced availability of GSH will affect the glyoxalase system and impair degradation of MG. This establishes a vicious cycle that leads to increased levels of MG. (Desai and Wu, 2008). A direct interaction of ALA and GSH has not been reported. However, in a recent study ALA given for 16 weeks to aging rats increased glutathione peroxidase and reduced oxidative stress (Guo *et al.*, 2009). GSH was not

measured in the study by Guo *et al*. (2009). ALA, by scavenging MG, can potentially prevent the interaction between MG and GSH. Thus, ALA can prevent the decrease in GSH caused by MG that was observed in our study. An increased availability of GSH in the ALA treated group can potentially lead to increased degradation of MG by the glyoxalase system with a consequent increase in D-lactate levels. This mechanism can explain the increase in plasma and aortic D-lactate levels that was found in MG+ALA treated groups. The elevated D-lactate levels observed in MG alone treated groups can be explained by increased metabolism of MG by the glyoxalase system until the later gets saturated.

In chronically fructose-fed SD rats, the serum MG levels are elevated to around 4 μM along with development of insulin resistance like syndrome (Jia and Wu, 2007). This raises an important question of whether MG is the cause or the effect of type 2 diabetes mellitus. Glucose and fructose are the major precursors of MG formation (Dhar *et al.*, 2008; Thornalley, 1996; Desai and Wu, 2007). Thus, a regular high intake of carbohydrates in normal people can result in increased MG formation, which can eventually lead to the development of insulin resistance and type 2 diabetes mellitus. Our results with acute MG-17 i.p. and the subsequent impaired glucose tolerance (Fig. 6-4A, B) *in vivo* point to the beginnings of insulin resistance. This theory gains weight in that MG-50 i.v. results in higher plasma MG levels and causes a greater impairment of glucose tolerance (Fig. 6-4C, D). Adipose tissue isolated from rats treated *in vivo* with MG-17 i.p. and MG-50 i.v. shows reduced insulin-stimulated glucose uptake (Fig. 6-5). These results provide further insight into the mechanisms behind the *in vivo* observations. In adipose tissue glucose transport is insulin-dependent and is mediated by GLUT 4. The acute effects of MG that might have an implication for the development of insulin resistance and diabetes have mostly been studied *in*

vitro in cultured cells. Thus, incubation of cultured 3T3-L1 adipocytes with MG (20 μM) reduced glucose uptake, decreased insulin-induced insulin-receptor substrate-1 (IRS-1) tyrosine phosphorylation, and decreased the activity of phosphatidylinositol 3-kinase (PI3K) (Jia and Wu, 2007). Incubation of cultured L6 muscle cells with high concentrations of MG (2.5 mM) for 30 min impaired insulin signaling (Riboulet-Chavey *et al.*, 2006). Incubation of insulin with MG modifies the structure of the insulin molecule in a way that impairs insulin-mediated glucose uptake in adipocytes (Jia *et al.*, 2006). To the best of our knowledge the effects of acute MG *in vivo* on glucose tolerance have not been reported previously. In a genetic model of diabetes such as the Zucker obese rat a defect of glucose transport in muscle has been reported (Sherman *et al.*, 1988). Protein kinase Akt2 (protein kinase B) plays a vital role in insulin signaling in muscle and liver and mice lacking Akt2 develop insulin resistance and a diabetes mellitus-like syndrome (Cho *et al.*, 2001). Our study reveals reduced insulin-mediated glucose uptake in adipose tissue from MG treated group, which may possibly be due to reduced GLUT4 mediated glucose uptake into the cells as indicated by reduced GLUT4 protein expression (Fig. 6-7). One or more steps in the insulin signaling pathway may also be impaired as indicated by reduced IRS-1 tyrosine phosphorylation (Fig. 6-9) (Jia and Wu, 2007; Baynes and Thorpe, 1999; Cho *et al.*, 2001; Birnbaum, 2001). Along with plasma glucose, the plasma insulin AUC was significantly higher after i.v. glucose load in the MG treated rats than its control group (Fig. 6-6B, D), indicating insulin resistance. ALA pretreatment attenuated the acute effects of MG on glucose tolerance which cannot be due to the AGEs cross-link breaking property of ALA since AGEs are formed by a slow process of reactions of MG with certain proteins that ranges from more than 24 h to many weeks (Desai and Wu, 2007; Thornalley, 1996). The attenuation of acute effects of MG seems to be most

likely due to binding of ALA with MG.

Plasma MG levels remained significantly elevated for at least 2 hours after a single intravenous or intraperitoneal injection of MG (Fig. 2) indicating a long half-life of more than 10 h (data not shown), which may lead to cumulative toxicity when MG is given daily (Slavik *et al.*, 1983). We have established doses for intraperitoneal and intravenous administration of MG that result in pathologically relevant concentrations in the plasma (Fig. 6-2).

In recent years western diet has increasing amounts of carbohydrates, and the rapid rise in the incidence of childhood obesity and type 2 diabetes mellitus has become a major health concern (Birnbaum, 2001; Van Dam *et al.*, 2002). In the absence of a genetic predisposition the link between high carbohydrate intake and the development of type 2 diabetes mellitus is unknown from a mechanistic perspective (Van Dam *et al.*, 2002). Carbohydrates are a major metabolic source of MG (Dhar *et al.*, 2008; Thornalley, 1996; Desai and Wu, 2007) and it would be interesting to examine the effects of ALA on chronic administration of high glucose or MG and the development of insulin resistance. The attenuation of acute effects of MG on glucose tolerance by ALA can be a promising strategy to prevent the chronic harmful effects of high glucose intake.

Conclusions

In summary, we have achieved pathologically relevant plasma levels of MG in normal SD rats using acute administration of exogenous MG through i.p. or i.v. route. The elevated MG induces glucose intolerance. ALA attenuates these effects of MG, an acute *in vivo* effect of ALA against MG, possibly due to scavenging, shown for the first time. Our study suggests a pathogenetic mechanism linking high carbohydrate intake and development of glucose

intolerance through increased formation of MG.

Acknowledgements

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CHAPTER 7

METHYLGLYOXAL SCAVENGERS ATTENUATE METHYLGLYOXAL AND HIGH GLUCOSE INDUCED ENDOTHELIAL DYSFUNCTION

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Summary

Background and purpose: Endothelial dysfunction is a feature of hypertension and diabetes. Methylglyoxal (MG) is a reactive dicarbonyl glucose metabolite. MG levels are elevated in spontaneously hypertensive rats and in diabetic patients. We investigated if MG induces endothelial dysfunction and whether MG scavengers can prevent MG and high glucose-induced endothelial dysfunction.

Experimental approach: We used isolated aortic rings from Sprague-Dawley rats for endothelium-dependent relaxation studies. We used cultured rat aortic and human umbilical vein endothelial cells. MG was measured by HPLC. Western blotting and assay kits were used.

Key results: Incubation of aortic rings with MG (30 μM) or high glucose (25 mM) significantly attenuated endothelium-dependent acetylcholine-induced relaxation, which was restored by the two different MG scavengers, aminoguanidine (100 μM) and N-acetyl cysteine (NAC, 600 μM). Treatment of cultured endothelial cells with MG or high glucose significantly increased cellular MG levels to a similar extent, prevented by aminoguanidine and NAC. In cultured endothelial cells MG and high glucose reduced basal and bradykinin-stimulated nitric oxide (NO) production, cyclic guanosine monophosphate levels, and serine-1177 phosphorylation and activity of endothelial nitric oxide synthase (eNOS) without affecting threonine-495 and Akt phosphorylation, and total eNOS protein. These effects of MG and high glucose were attenuated by aminoguanidine or NAC.

Conclusions and implications: Our results show for the first time that MG reduces serine-1177 phosphorylation and activity of eNOS, reduces NO production, and causes endothelial dysfunction similar to high glucose. Specific and safe MG scavengers have the potential to

prevent MG and high glucose-induced endothelial dysfunction.

Keywords: Methylglyoxal, eNOS, hyperglycemia, endothelial dysfunction, aminoguanidine, N-acetyl cysteine, methylglyoxal scavengers

Introduction

Endothelial dysfunction, commonly defined as reduced endothelium-dependent vascular relaxation, occurs as an early event in atherosclerosis, hypertension (O'Keefe *et al.*, 2009), the prediabetic stage of insulin resistance (Su *et al.*, 2008), and is a hallmark of type 2 and type 1 diabetes mellitus (De Vriese *et al.*, 2000, Potenza *et al.*, 2009). Nitric oxide (NO) is one of the main vasodilator mediators released from the endothelium (Palmer *et al.*, 1987). It is synthesized by endothelial nitric oxide synthase (eNOS) from L-arginine with L-citrulline as a co-product (Palmer *et al.*, 1988). NO has a short half-life (6-7 s). Reduced production or availability of NO is a common feature of endothelial dysfunction (De Vriese *et al.*, 2000, Potenza *et al.*, 2009).

Methylglyoxal (MG) is a highly reactive dicarbonyl metabolite produced during glucose metabolism (Desai and Wu, 2007). The clinical significance of MG lies in the fact that it reacts with and modifies certain proteins, lipids and DNA and alters their normal structure and/or function (Desai and Wu, 2007, Baynes JW Thorpe SR, 1999). MG is a major precursor of advanced glycation endproducts (AGEs), which are implicated in the pathogenesis of vascular complications of diabetes (Desai and Wu, 2007, Baynes JW Thorpe SR, 1999). We have shown earlier that MG levels are elevated in spontaneously hypertensive rats (Wang *et al.*, 2005), in fructose-fed hypertensive rats (Wang *et al.*, 2008), and in diabetic patients (Wang *et al.*, 2007a). We have also shown that incubation of vascular smooth muscle cells (VSMCs) with 25 mM glucose or fructose for 3 h increases MG production 3.5 or 3.9 fold, respectively, and increases oxidative stress (Dhar *et al.*, 2008). The aim of the current study was to examine if MG induces endothelial dysfunction and the mechanism involved. Even though high glucose has previously been shown to cause endothelial dysfunction (Potenza *et al.*, 2009,

Triggle, 2008, Nishikawa *et al.*, 2000, Du *et al.*, 2001) we performed parallel experiments with high glucose to see if the functional and molecular changes produced by MG and high glucose are similar. We examined whether two different MG scavengers, *viz*. aminoguanidine (Lo *et al.*, 1994, Wang *et al.*, 2007b) and N-acetyl cysteine (NAC) (Vasdev *et al.*, 1998, Jia and Wu, 2007), can prevent any deleterious effects of MG and high glucose on endothelial function.

Materials and Methods

Animals

Male 11-week old Sprague-Dawley (SD) rats from Charles River Laboratories (Quebec, Canada) were used according to a protocol approved by the Animal Care Committee at The University of Saskatchewan (Protocol No. 20070029), following guidelines of the Canadian Council on Animal Care. The rats were acclimatized for one week. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Isometric tension studies on aortic rings

A group of 24 SD rats was used. Isometric tension studies were carried out on rat aortic rings as described (Wu *et al.*, 1998). Briefly, 3-4 mm thoracic aortic rings from SD rat were mounted under a 2 g load in four separate 10 mL organ baths containing Krebs solution with 5 mM glucose and maintained at 37° C and bubbled with 95% O_2 + 5% CO_2. After a 90 min equilibration period the rings were pre-contracted with phenylephrine (1 μM) and cumulative concentration-dependent relaxation in response to acetylcholine (ACh) was

obtained before (Control) and 2 h after incubation with either glucose (15 or 25 mM) or MG (30 or 100 μM). In initial experiments the responses to ACh were repeated before and 2 h after incubation with normal Krebs solution to confirm reproducibility of responses to ACh. Some sets of rings were co-incubated with the MG scavenger aminoguanidine (AG, 100 μM) (Lo *et al.*, 1994, Wang *et al.*, 2007b), or another MG scavenger, NAC (600 μM) (Vasdev *et al.*, 1998, Jia and Wu, 2007), or the NADPH oxidase inhibitor apocynin (100 μM) for 2 h. Treatment with each compound was tested in rings from at least 5 different rats. Isometric tension was measured with isometric force transducers with the 'Chart' software and Powerlab equipment (AD Instruments Inc., Colorado Springs, CO, USA).

Cell culture

Rat aortic endothelial cells (RAECs) were isolated from male SD rats according to the method of McGuire *et al* (McGuire and Orkin, 1987). The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 0.15 mg/mL endothelial cell growth supplement (Biomedical Technologies Inc., MA, USA). For the initial culture Matrigel^{TM} (Sigma-Aldrich, Oakville, ON, Canada) coated culture dishes were used. RAECs were identified by their typical cobblestone morphology and positive staining for von Willebrand factor. Immunostaining was done as described by us earlier (Dhar *et al.*, 2008). Cells between passage 3 and 6 were used for the experiments. Human umbilical vein endothelial cells (HUVECs) from American Type Culture Collection were cultured in Kaighns F12K medium containing 10% fetal bovine serum (FBS), 0.1 mg/mL heparin and 0.03-0.05 mg/mL endothelial cell growth supplement.

Nitric oxide assay

Confluent cells were washed twice with Hanks balanced salt solution (HBSS) and incubated with MG (3, 10 or 30 μM) or glucose (15 or 25 mM) in HBSS for 3 or 24 h at 37º C in an incubator. The supernatant was analyzed for basal NO production. The cells were further incubated with bradykinin (BK, 10 μM), an endothelial cell agonist for NO production (Palmer *et al.*, 1987), for 15 min and the supernatant was collected and levels of nitrate plus nitrite were measured with the Griess assay kit (Caymen Chemicals, Ann Arbor, MI, USA) (Dhar *et al.*, 2008). Nitrate in the sample was first converted to nitrite by nitrate reductase. One set of HUVECs was also co-incubated with AG (100 μM) or NAC (600 μM), and MG (30 μM) or glucose (25 mM) for 24 h following which the basal and BK-stimulated NO production were measured.

Cyclic guanosine monophosphate (cGMP) assay

Briefly, control and test compounds treated cells were treated with the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 100 μM), for 30 min before agonist stimulation. Cells were harvested into the supplied lysis buffer and subjected to cGMP measurement using the cGMP assay kit (R & D Systems, MN, USA) according to the manufacturer's protocol. To normalize cGMP values, protein content in each dish was measured by the BCA Protein assay (Bio-Rad, Hercules, CA, USA).

Methlyglyoxal assay

MG was measured by a specific and sensitive HPLC method as described before (Dhar *et al.*, 2008, Dhar *et al.*, 2009). MG was derivatized with *o*-phenylenediamine (*o*-PD) to form

the specific quinoxaline product, 2-methylquinoxaline. The samples were incubated in the dark for 24 h with 0.45 N perchloric acid and 10 mM *o*-PD at room temperature. The quinoxaline derivatives of MG (2-methylquinoxaline) and the quinoxaline internal standard (5-methylquinoxaline) were quantified on a Hitachi D-7000 HPLC system (Hitachi, Ltd., Mississauga, ON, Canada) *via* Nova-Pak® C18 column (3.9 ×150 mm, and 4 μm particle diameter, Waters, MA, USA).

eNOS activity assay

The NOS activity assay is based on the biochemical conversion of [^{3}H] L-arginine to [^{3}H] L-citrulline by NOS. Briefly, control and test compounds treated cells were washed in PBS, harvested and centrifuged for 2 min to pellet the cells. The cells were resuspended in 1x homogenization buffer and sonicated briefly. The suspension was centrifuged, the supernatant was separated and the resulting protein concentration was adjusted to 5-10 mg/mL. The eNOS activity was measured using the Cayman Chemicals NOS activity assay kit (Cayman Chemical Company, MI, USA).

Measurement of reduced glutathione (GSH)

Briefly, monochlorobimane stock (100 μM) was added to endothelial cells treated with test compounds. After 30 min, the medium was collected for medium GSH measurement. Cells were washed with PBS and harvested in 1 mL of 1% sodium dodecylsulfate (SDS) in 50 mM Tris buffer (pH 7.5), sonicated and the aliquots (100 μL) of supernatants were read in triplicate with an excitation wavelength of 380 nm and an emission wavelength of 470 nm (Wu and Juurlink, 2002, Kamencic *et al.*, 2000).

Western blotting

Cell lysates were prepared as described earlier (Jia and Wu, 2007, Wu and Juurlink, 2002) and the protein concentration in the supernatant was determined by the BCA Protein assay (Bio-Rad, Hercules, CA, USA). Aliquots of cell lysates (50 µg of protein each) were separated on 7.5-10% SDS-PAGE, electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad), blocked with 5% nonfat milk in TBS-Tween buffer for 1.5 h at room temperature, and incubated overnight at 4° C with the primary antibody, eNOS and phospho-NOS-1177 (both from BD Transduction Laboratories, Mississauga, ON, Canada), phospho-NOS-495 and GSH-reductase (both from Santa Cruz, CA, USA), anti-Akt, anti-phos-Akt (Cell Signaling, MA, USA), and then with horseradish peroxidase conjugated secondary antibody (Santa Cruz, CA, USA) for 1 h at room temperature. After extensive washing, the immunoreactive proteins were detected with an Enhanced Chemiluminescence Detection System (ECL; Amersham Biosciences Corp., Piscataway, NJ, USA) (Jia and Wu, 2007, Wu and Juurlink, 2002).

Measurement of reactive oxygen species and NADPH oxidase activity

Confluent cells were loaded with a membrane-permeable, nonfluorescent probe 2',7'-dichlorofluorescin diacetate (CM-H2DCFDA, 5 µM) for 2 h at 37° C in FBS-free medium in the dark. The cells treated with MG (30 µM) or glucose (25 mM) for 24 h were assayed for fluorescent oxidized dichlorofluorescein (DCF) as an indicator of production of reactive oxygen species (ROS) as described earlier (Dhar *et al.*, 2008), and for activity of NADPH oxidase (Griendling *et al.*, 1994), which is a key endothelial cell enzyme for the production of

superoxide anions (Gao and Mann, 2009). The protein content of the homogenate was measured by BCA Protein assay (Bio-Rad, Hercules, CA, USA). NADPH oxidase activity was measured by a luminescence assay in a 50-mmol/L phosphate buffer, pH 7.0, containing 1 mmol/L EGTA, 150 mmol/L sucrose, 500 µmol/L lucigenin as the electron acceptor, and 100 µmol/L NADPH as the substrate (final volume, 0.9 mL) (Griendling *et al.*, 1994).

Chemicals and Statistical analysis

All chemicals were of analytical grade. Methylglyoxal, D-glucose, N-acetyl cysteine, apocynin and glutathione were purchased from Sigma Aldrich, Oakville, ON, Canada. Cell culture media and reagents were purchased from Invitrogen Canada Inc., Burlington, ON, Canada. Data are expressed as mean ± SEM and analyzed using one way ANOVA and *post hoc* Bonferroni's test. The *P* value was considered significant when it was less than 0.05.

Results

Methylglyoxal and high glucose reduce acetylcholine-induced relaxation of aortic rings: attenuation by MG scavengers

In rat aortic rings precontracted with phenylephrine (1 µM), MG (30 or 100 µM) incubated for 2 h in the bath caused significant inhibition of ACh-induced endothelium-dependent relaxation, which was prevented by coincubation of AG (100 µM) with MG (100 µM) (Fig. 7-1A). AG (10 and 30 µM) did not prevent MG (100 µM)-induced reduction of relaxation (data not shown). High glucose (15 and 25 mM) incubated for 2 h also attenuated ACh-induced relaxation of rat aortic rings that was prevented by coincubation of glucose (25 mM) with AG (100 µM) (Fig. 7-1B). MG (30 µM) and glucose (25 mM) induced attenuation

of relaxation was also restored by another MG scavenger, NAC (600 μM) (Vasdev *et al.*, 1998, Jia and Wu, 2007) (Fig. 7-1C, D). However, MG and high glucose-induced attenuation of relaxation was not restored by the NADPH oxidase inhibitor apocynin (100 μM) (Fig. 7-1E, F). In washout experiments on aortic rings the reduced relaxation induced by MG (30 μM) and glucose (25 mM) for 2 h was restored after a further 2 h washout with changes of Krebs solution in the bath every 15 min (data not shown). MG and glucose did not affect endothelium-independent relaxation of aortic rings induced by sodium nitroprusside (data not shown). In preliminary experiments MG (3 and 10 μM) or the osmotic control mannitol (25 mM) incubated for 2 h did not affect ACh-induced relaxation (data not shown).

High glucose and exogenous methylglyoxal increase methylglyoxal levels in endothelial cells

Incubation of RAECs and HUVECs with MG (30 or 100 μM) for 24 h significantly increased the level of cellular MG that was prevented by coincubation with AG (100 μM) or NAC (600 μM) (Fig. 7-2A, C). Incubation of cultured RAECs and HUVECs with glucose (25 mM) for 24 h also significantly increased MG levels in these cells (Fig. 7-2B, D), which was also prevented by coincubation of HUVECs with AG (100 μM) or NAC (600 μM) and glucose (25 mM) for 24 h (Fig. 7-2B, D). Incubation of RAECs and HUVECs with 25 mM glucose for 3 h also significantly increased cellular MG levels (data not shown).

The increase in cellular MG in RAECs and HUVECs induced by glucose (25 mM) and exogenous MG (30 μM) was similar (Fig. 7-2).

Methylglyoxal and high glucose reduce nitric oxide production in RAECs and HUVECs

Incubation of RAECs and HUVECs with 3, 10 or 30 μM MG for 3 or 24 h decreased basal and BK (10 μM)-stimulated NO production in both cell types to varying degrees depending on the concentration of MG and the incubation time (Figs. 7-3A, C & 7-4A, C). The inhibition of basal and agonist-stimulated NO production was significant with 30 μM MG incubated for 24 h in both RAECs and HUVECs (Figs. 7-3C and 7-4C). The attenuation of basal and BK-stimulated NO production by MG (30 μM) incubated for 24 h was restored by coincubation with AG (100 μM) or NAC (600 μM) (Fig. 7-3C and 7-4C).

Similarly, incubation of RAECs and HUVECs with glucose (15 or 25 mM for 3 or 24 h) decreased basal and BK (10 μM)-stimulated NO production to varying degrees depending on the concentration of glucose and the incubation time (Figs. 7-3B, D and 7-4B, D). The inhibition of basal and agonist-stimulated NO production in both cell types was significant with 25 mM glucose incubated for 24 h (Figs. 7-3D and 7-4D). The attenuation of basal and BK-stimulated NO production by glucose (25 mM) incubated for 24 h was restored by coincubation with AG (100 μM) or NAC (600 μM) (Fig. 7-3D and 7-4D). AG (100 μM) alone for 24 h did not affect basal or BK-stimulated NO production (data not shown).

Methylglyoxal and high glucose reduce agonist-stimulated cGMP increase in RAECs and HUVECs

cGMP is the second messenger of NO-induced activation of soluble guanylate cyclase and is a sensitive indicator of NO production (Waldman and Murad, 1987, Papapetropoulos *et al.*, 1996). Incubation of RAECs and HUVECs with 30 μM MG or 25 mM glucose for 24 h prevented BK (10 μM)-stimulated cGMP increase in both cell types that was restored by coincubation with AG (100 μM) or NAC (600 μM) (Fig. 7-5A, B).

Methylglyoxal and high glucose reduce activity of the eNOS enzyme

To understand the mechanism of reduced NO production with MG and high glucose, the activity of the eNOS enzyme was tested in an eNOS activity assay. Incubation of RAECs (data not shown), as well as HUVECs, with MG (30 μM) or glucose (25 mM) for 24 h reduced NO production catalyzed by eNOS in the eNOS activity assay, which was prevented by coincubation with AG (100 μM) (Fig. 7-6A). At the same time both MG and high glucose did not affect the eNOS protein level in HUVECs (Fig. 7-6B) or RAECs (data not shown) under the same treatment conditions indicating that the reduced NO production from RAECs and HUVECs by MG and glucose was due to reduced activity of the eNOS enzyme and not due to reduced eNOS protein expression.

Methylglyoxal and high glucose reduce bradykinin-stimulated serine-1177 phosphorylation of the eNOS enzyme

To further elucidate the mechanism of reduced eNOS activity by MG and high glucose, we examined the serine-1177 and threonine-495 phosphorylation of eNOS, and phsophorylation of Akt, which is a substrate for serine 1177 of eNOS (Fulton *et al.*, 1999, Dimmeler *et al.*, 1999). In HUVECs, treatment with MG (30 μM) or glucose (25 mM) for 24 h reduced BK-stimulated serine-1177 phosphorylation of eNOS that was prevented by co-incubation with AG (100 μM) (Fig. 7-6C). There was little basal serine-1177 phosphorylation of eNOS, which was almost abolished by MG (30 μM) or glucose (25 mM) incubated for 24 h. The antibody for serine-1177 phosphorylated eNOS did not react well with RAEC eNOS and hence that data is not shown. eNOS was phosphorylated basally at threonine-495. BK

stimulation reduced the threonine-495 phosphorylation of eNOS (Fig. 7-6C). MG (30 μM) or glucose (25 mM) incubated for 24 h did not affect basal or BK-stimulated threonine-495 phosphorylation of eNOS (Fig. 7-6C). MG and high glucose also did not affect phosphorylation of Akt (Fig. 7-6C).

Methylglyoxal and high glucose increase oxidative stress in HUVECs and RAECs

Incubation of cultured HUVECs and RAECs with MG (30 μM) or glucose (25 mM) for 24 h caused a significant increase in oxidized DCF, an indicator of ROS production, in both cell types which was attenuated by coincubation with NAC (600 μM) (Fig. 7-7A, B). Incubation of RAECs and HUVECs with MG (30 μM) or glucose (25 mM) for 24 h, also caused a significant increase in the activity of NADPH oxidase, as measured by superoxide anion production in an activity assay, that was prevented by the NADPH oxidase inhibitor apocynin, in both cell types (Fig. 7-7C, D).

Methylglyoxal and high glucose decrease GSH levels and glutathione reductase protein in RAECs and HUVECs

MG is degraded by the glyoxalase enzymes that use GSH as a cofactor. Incubation of HUVECs (Fig. 7-8A) and RAECs (data not shown) with MG (30 μM) or glucose (25 mM) for 24 h significantly reduced GSH levels and also decreased glutathione reductase protein expression, which was prevented by coincubation with AG (100 μM) in HUVECs (Fig. 7-8B).

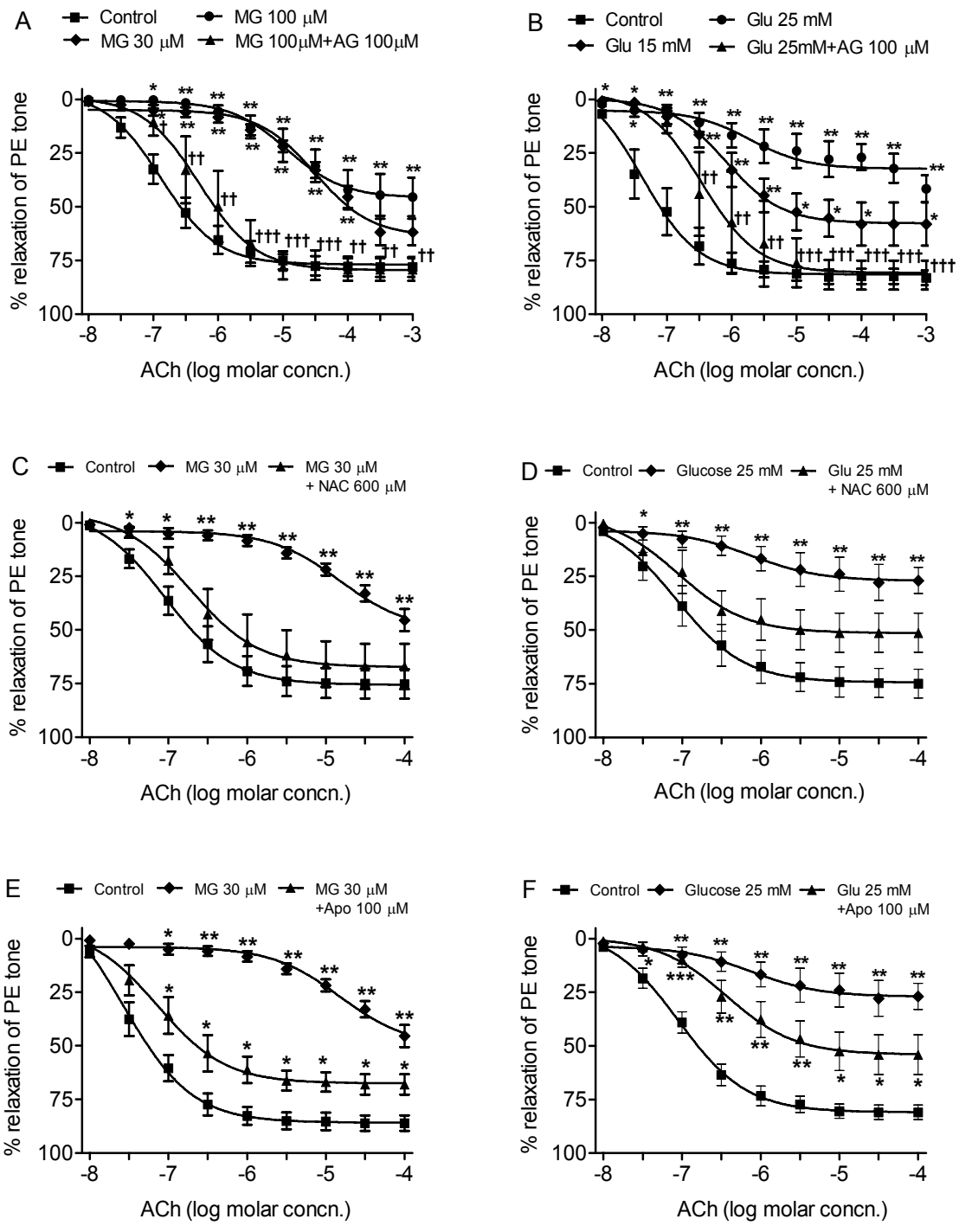

Figure 7-1 Methylglyoxal (MG) scavengers attenuate MG and high glucose induced endothelial dysfunction in isolated aortic rings from Sprague-Dawley rats. Dose-related

responses were obtained to acetylcholine (ACh) in phenylephrine (PE, 1 μM) precontracted rings before (control) and 2 h after incubation with (A, C, E) MG (30 or 100 μM) or (B, D, F) glucose (Glu, 15 or 25 mM). In some sets of rings the MG scavenger aminoguanidine (AG, 100 μM) was co-incubated with (A) MG (100 μM) or (B) glucose (25 mM); the MG scavenging antioxidant N-acetyl cysteine (NAC 600 μM) was co-incubated with (C) MG (30 μM) or (D) glucose (25 mM); or the NADPH oxidase inhibitor apocynin (Apo 100 μM) was co-incubated with (E) MG (30 μM) or (F) glucose (25 mM) for 2 h. (n = 5 rings from different rats for each test compound). *P<0.05, **P<0.01 vs corresponding control value, †P<0.05, ††P<0.01, †††P<0.001 vs corresponding (A) MG 100 μM value or (B) glucose (25 mM).

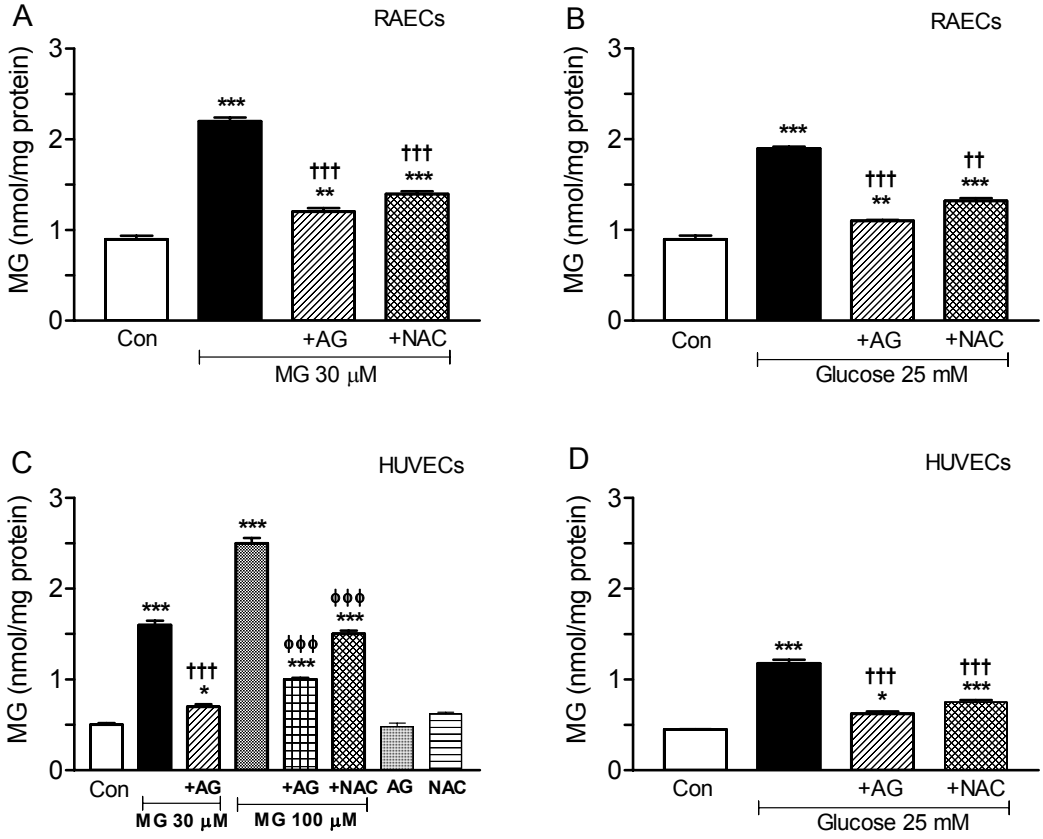

Figure 7-2 High glucose and exogenous methylglyoxal (MG) increase cellular MG levels in cultured endothelial cells: attenuation by MG scavengers. Confluent rat aortic endothelial cells (RAECs) and human umbilical vein endothelial cells (HUVECs) were incubated with normal culture medium (Control, Con) or medium containing (A, C) MG (30 or 100 μM), or (B, D) glucose (Glu, 25 mM), for 24 h. Aminoguanidine (AG, 100 μM) or N-acetyl cysteine (600 μM) was coincubated with (A, C) MG (30 or 100 μM), or with (B, D) glucose (25 mM) for 24 h. Cellular MG was measured by HPLC. n = 4 for each treatment. *P<0.05, **P<0.01, ***P<0.001 vs corresponding control value, ††P<0.01, †††P<0.001 vs corresponding (A, C) MG (30 μM), or (B, D) glucose (25 mM) value, (C) $^{\varphi\varphi\varphi}$P<0.001 vs MG 100 μM.

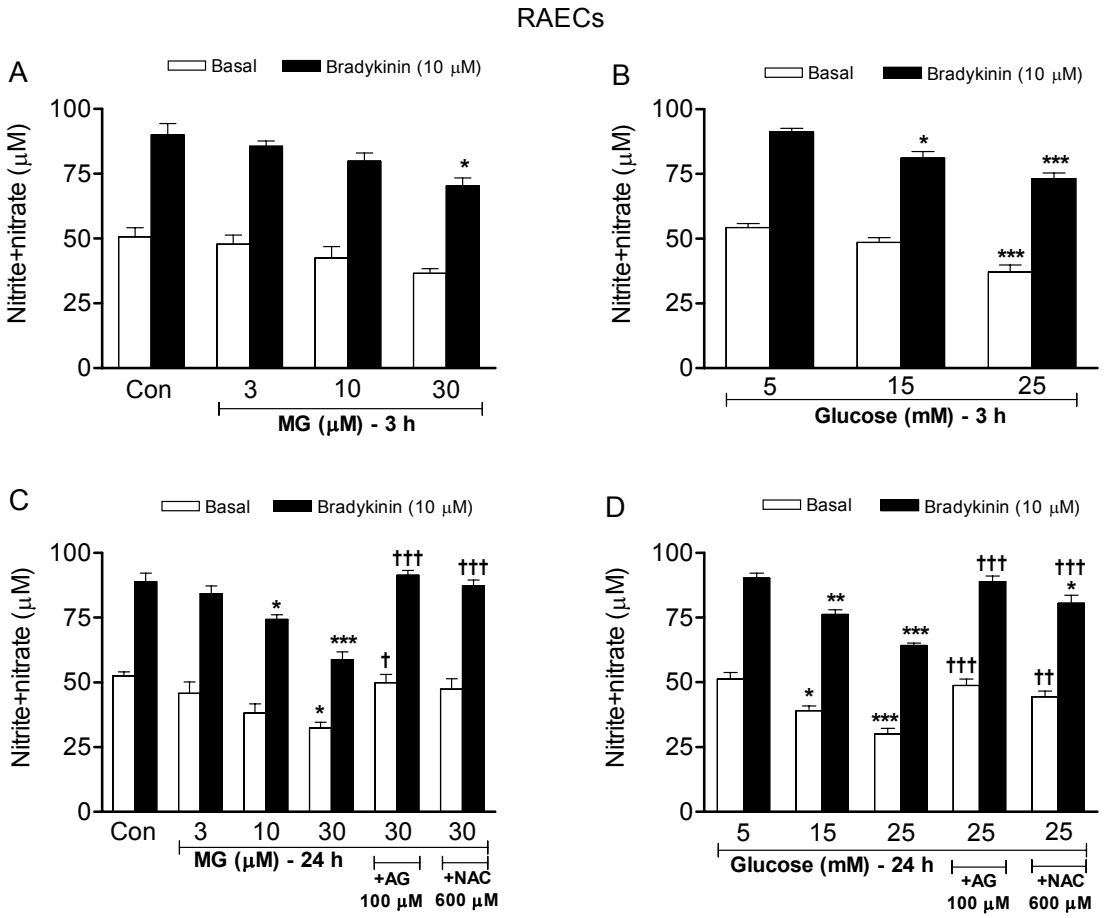

Figure 7-3 Methylglyoxal (MG) and high glucose reduce nitric oxide production in cultured rat aortic endothelial cells (RAECs). RAECs were incubated with (A, C) MG (3, 10 or 30 μM), or (B, D) glucose (5, 15 or 25 mM) for (A, B) 3 h or (C, D) 24 h. Cells were coincubated with (C) MG (30 μM) or (D) glucose (25 mM) and aminoguanidine (AG, 100 μM) or N-acetyl cysteine (NAC, 600 μM) for 24 h. The supernatant was collected after the 3 or 24 h incubation time (basal) and the cells were further incubated with bradykinin (10 μM) for 15 min to stimulate nitric oxide production and the supernatant was analyzed for nitrite+nitrate levels by the Griess assay. n = 8 for each group. *P<0.05, **P<0.01, ***P<0.001 vs corresponding (A, C) control value or (B, D) glucose (5 mM). †P< 0.05, ††P< 0.01, †††P< 0.001 vs corresponding

(C) MG (30 μM) alone or (D) glucose (25 mM) value.

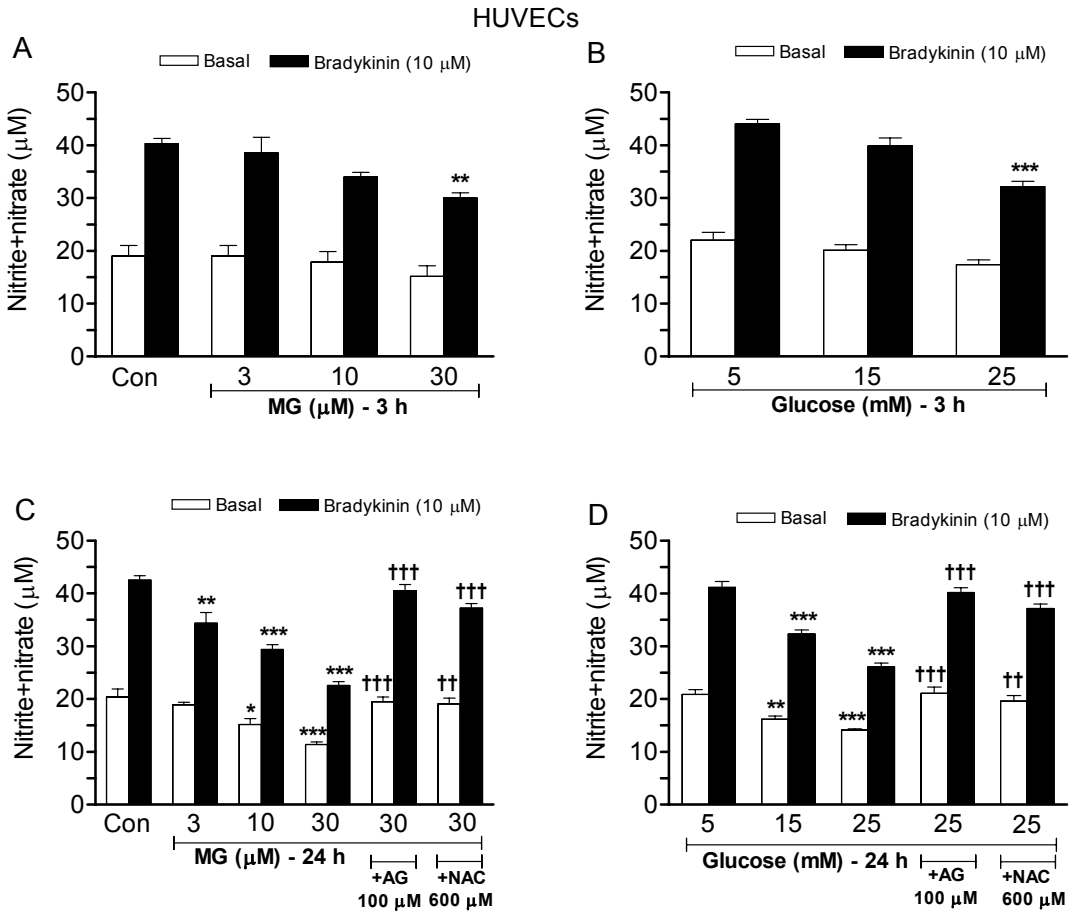

Figure 7-4 Methylglyoxal (MG) and high glucose reduce nitric oxide production in cultured human umbilical vein endothelial cells (HUVECs). HUVECs were incubated with (A, C) MG (3, 10 or 30 μM), or (B, D) glucose (5, 15 or 25 mM) for (A, B) 3 h or (C, D) 24 h. Aminoguanidine (AG, 100 μM) or N-acetyl cysteine (NAC, 600 μM) was coincubated with (C) MG (30 μM), or with (D) glucose (25 mM) for 24 h in some sets of cells. The supernatant was collected after the 3 or 24 h incubation time (basal) and the cells were further incubated with bradykinin (10 μM) for 15 min to stimulate nitric oxide production and the supernatant was analyzed for nitrite+nitrate levels by the Griess assay. n = 8 for each group. *P<0.05, **P<0.01, ***P<0.001 vs corresponding (A, C) control value or (B, D) glucose 5 mM.

$^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ vs. corresponding (C) MG (30 μM) alone value or (D) glucose (25 mM) alone.

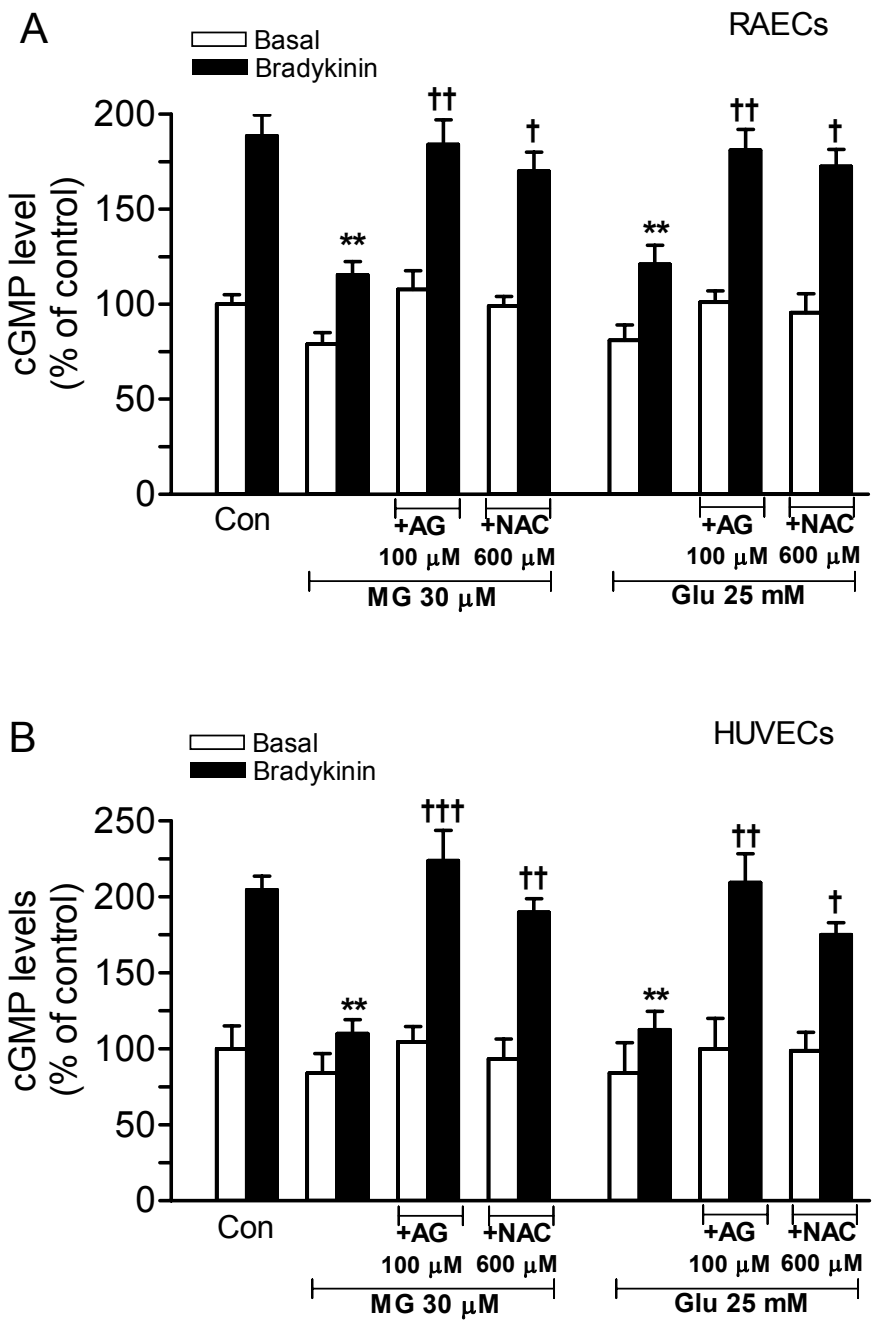

Figure 7-5 Methylglyoxal (MG) and high glucose reduce cyclic guanosine monophosphate (cGMP) production in cultured rat aortic endothelial cells (RAECs) and human umbilical vein endothelial cells (HUVECs). (A) RAECs and (B) HUVECs were incubated with MG (30 μM) or glucose (25 mM) for 24 h. Aminoguanidine (AG, 100 μM) or N-acetyl cysteine (NAC, 600 μM) was coincubated with MG (30 μM), or with glucose (25 mM) for 24 h in some sets of

cells. Basal and bradykinin (BK, 10 μM)-stimulated cGMP levels were measured with an assay kit as described in the methods. n = 8 for each group. **P<0.01 *vs* corresponding control value. $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01, $^{\dagger\dagger\dagger}P$<0.001 vs. corresponding MG (30 μM) alone or glucose (25 mM) alone value.

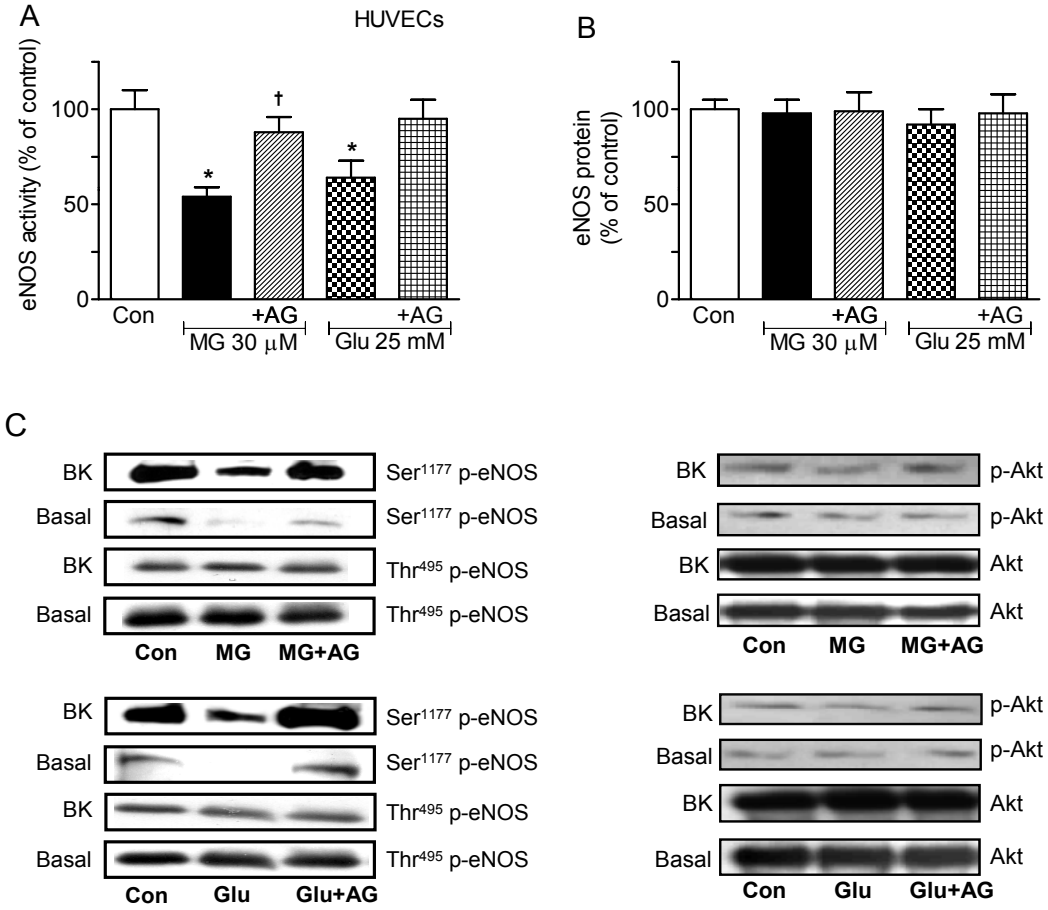

Figure 7-6 Methylglyoxal (MG) and high glucose reduce serine-1177 phosphorylation and activity of endothelial nitric oxide synthase (eNOS). Human umbilical vein endothelial cells (HUVECs) were incubated with MG (30 μM) alone or glucose (Glu, 25 mM) alone, or coincubated with aminoguanidine (AG, 100 μM) for 24 h following which an eNOS activity assay was performed, based on conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline, using an activity assay kit (A). (B) The total eNOS protein was determined in the same treated cells from (A) by western blotting. (C) Basal and bradykinin (BK, 10 μM)-stimulated serine-1177, and threonine-495 phosphorylation of eNOS; and Akt and phosphorylated Akt were determined with appropriate anti-phospho-eNOS, anti-Akt and anti-phospho-Akt antibodies in

cells treated as in (A) by western blotting. n = 5 for each group. *P<0.05 vs. control, †P<0.05 vs. MG 30 μM.

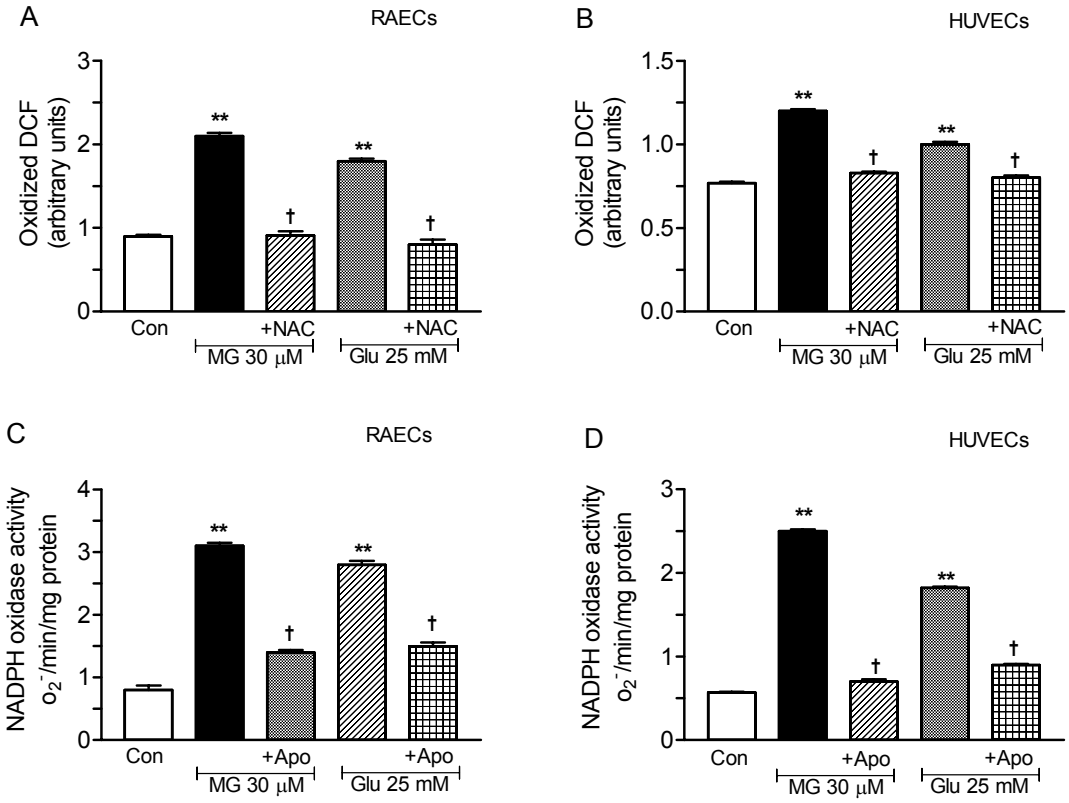

Figure 7-7 Methylglyoxal (MG) and high glucose increase reactive oxygen species production and NADPH oxidase activity in cultured endothelial cells. Incubation of cultured (A, C) rat aortic endothelial cells (RAECs) and (B, D) human umbilical vein endothelial cells (HUVECs) with MG (30 μM) or glucose (25 mM) for 24 h increased (A, B) reactive oxygen species (ROS) production, measured as oxidized dichlorofluorescein (DCF), and (C, D) NADPH oxidase activity that was prevented by co-incubation with (A, B) the antioxidant N-acetylcysteine (NAC 600 μM) or (C, D) the NADPH oxidase inhibitor, apocynin (Apo, 100 μM), respectively. NADPH oxidase activity was measured with a luminescence assay. n = 5 for each group. **P<0.01 vs. corresponding control, †P<0.05 vs. corresponding glucose (25 mM) alone or MG (30 μM) alone value.

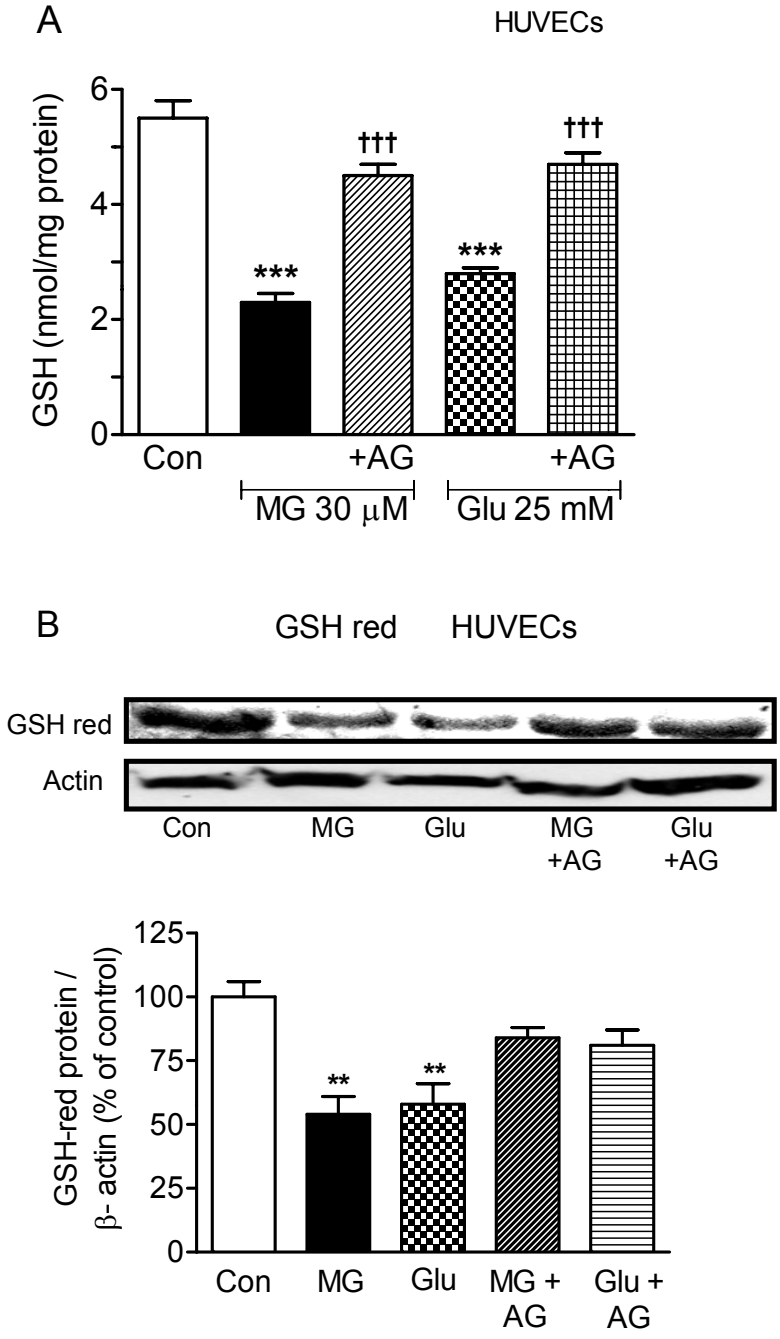

Figure 7-8 Methylglyoxal (MG) and high glucose decrease glutathione and glutathione reductase in cultured human umbilical vein endothelial cells (HUVECs). Incubation of HUVECs with MG (30 μM) or glucose (25 mM) for 24 h decreased (A) cellular reduced

glutathione (GSH) levels and (B) glutathione reductase (GSH red) protein levels that was prevented by co-incubation with aminoguanidine (AG, 100 μM). GSH was determined with the monochlorobimane assay as described in methods. n = 5 for each group. **P<0.01, ***P<0.001 vs. corresponding control, †††P<0.001 vs. corresponding MG (30 μM) alone, or glucose (25 mM) alone value.

Discussion

In this study we provide evidence, for the first time, that MG, a glucose metabolite, induces endothelial dysfunction in isolated rat aortic rings as well as in cultured RAECs and HUVECs. High glucose (25 mM) increases MG levels in both RAECs and HUVECs, and induces endothelial dysfunction in aortic rings and cultured endothelial cells similar to MG. The effects of MG and high glucose on aortic rings and cultured endothelial cells are attenuated by two different MG scavengers, aminoguanidine (Lo *et al.*, 1994, Wang *et al.*, 2007b) and NAC (Vasdev *et al.*, 1998, Jia and Wu, 2007). Thus, our results provide a possible mechanism linking high glucose and endothelial dysfunction. The effects of MG and high glucose to reduce eNOS activity, NO production, and increase oxidative stress are seen in endothelial cells of rat and human origin and hence are not limited to one species. We have recently shown that MG levels are comparatively higher in the aorta compared to other organs such as heart, liver, lungs, kidney etc., and after exogenous MG administration the aortic levels increased significantly (Dhar *et al.*, 2010).

To the best of our knowledge, the effect of MG on endothelium-dependent vascular relaxation has not been reported previously. Both, MG (30 and 100 μM) and high glucose (15 and 25 mM) reduced ACh-induced relaxation to a similar extent. In fact 25 mM glucose caused a slightly greater reduction of relaxation. It should be pointed out that exogenous MG is not fully absorbed into the cells. In one study as little as 3% of exogenous MG was absorbed by cultured L6 muscle cells incubated with 2.5 mM MG (Riboulet-Chavey *et al.*, 2006). As shown in Fig. 7-2, 30 μM MG causes a similar increase in cellular MG levels as 25 mM glucose in endothelial cells, which justifies the use of 30 μM exogenous MG in our study. The attenuation of relaxation by both, MG and high glucose, was prevented by two different

MG scavengers, aminoguanidine and NAC (Lo *et al.*, 1994, Wang *et al.*, 2007b, Vasdev *et al.*, 1998, Jia and Wu, 2007) (Fig. 7-1A-D), but not by the NADPH oxidase inhibitor apocynin (Fig. 7-1E, F), indicating MG as a possible mediator of the effects of high glucose on endothelial dysfunction.

The reduction of NO production by cultured endothelial cells treated with MG has not been reported previously. We measured the production of NO in both cell types as nitrate plus nitrite (Fig. 7-3 & 7-4), as well as cGMP accumulation (Fig. 7-5) in response to agonist stimulation. cGMP is a sensitive indicator of NO production, which activates soluble guanylate cyclase (Waldman and Murad, 1987, Papapetropoulos *et al.*, 1996). Again, both MG scavengers, aminoguanidine and NAC, prevented the reduced NO production by both MG and high glucose. It should be pointed out here that aminoguanidine is also an inhibitor of inducible NOS (Corbett *et al.*, 1992, Misko *et al.*, 1993) but iNOS is not normally expressed by endothelial cells. In the case that aminoguanidine was inhibiting eNOS it should further reduce the NO production along with MG and high glucose. In our study, aminoguanidine actually attenuated the reduction in NO production by MG and high glucose, suggesting a MG scavenging effect of aminoguanidine (Lo *et al.*, 1994, Wang *et al.*, 2007b). Moreover, we used a relatively lower concentration of aminoguanidine (100 μM) compared to concentrations of 1 mM and higher reported in the literature (Lo *et al.*, 1994, Misko *et al.*, 1993).

The mechanism of reduced NO production can be ascribed to reduced eNOS activity induced by MG and high glucose with no change in eNOS protein level (Fig. 7-6A, B). The reduced eNOS activity is likely due to reduced BK-stimulated serine-1177 [bovine eNOS serine-1179] phosphorylation of eNOS (Dimmeler *et al.*, 1999, Fleming *et al.*, 2001) caused by MG (30 μM) or glucose (25 mM) (Fig. 7-6C). Serine-1177 is the most common site

phosphorylated in activated eNOS (Fulton *et al.*, 1999, Dimmeler *et al.*, 1999). Basal eNOS is phosphorylated at threonine-495 and. BK stimulation causes dephosphorylation of threonine-495 [bovine eNOS threonine-497] (Fleming *et al.*, 2001). MG (30 μM) or glucose (25 mM) did not affect threonine-495 phosphorylation of e-NOS (Fig. 7-6C). High glucose (25 mM) has been reported to reduce serine-1179 (human serine-1177) phosphorylation of eNOS due to activation of inhibitor kappa β kinase (IKKβ), a mediator of inflammation, and reduce NO production in cultured bovine aortic endothelial cells (Kim *et al.*, 2005). Since serine-1177 is the phosphorylation site for the substrate Akt (Fulton *et al.*, 1999, Dimmeler *et al.*, 1999) we looked at Akt phosphorylation itself. MG and hyperglycemia did not affect Akt phosphorylation in HUVECs (Fig. 7-6C). Thus, reduced Akt phosphorylation is not responsible for reduced eNOS activity.

One study (Brouwers *et al.*, 2008) has reported a lack of effect of MG and MG-adducts (argpyrimidine and 5-hydro-5-methylimidazolone) (1, 10 and 100 μM of each) on eNOS activity in whole cell homogenates of HUVECs incubated with MG and its two adducts. Some possible reasons for this lack of effect of MG could be the incubation time of 60 min, and use of whole cell homogenates instead of intact cultured cells, which provides a different experimental condition. Moreover, the authors used 10 μmol/L of total free arginine and since MG has high affinity for arginine, it is possible that the added MG bound to arginine in the reaction mix and did not affect eNOS. In contrast, Du *et al.* (Du *et al.*, 2001) have reported that hyperglycemia inhibits eNOS activity by activating, the hexosamine pathway, increasing superoxide production and reducing serine-1177 phosphorylation of eNOS. We have shown that MG also reduces serine-1177 phosphorylation of eNOS and is a possible mediator of hyperglycemia-induced dysfunction.

Increased superoxide anion can quench NO to form peroxynitrite and thus reduce the bioavailability of NO (Pacher *et al.*, 2007). This is the more frequently reported mechanism of endothelial dysfunction caused by oxidative stress (Potenza *et al.*, 2009). Hyperglycemia (30 mM glucose) has been shown to induce endothelial dysfunction by increasing production of ROS, oxidative stress and activating protein kinase C and NFκB (Potenza *et al.*, 2009, Triggle, 2008, Nishikawa *et al.*, 2000). However, in these studies it was not shown if high glucose *per se* or one of its metabolites is responsible for causing endothelial dysfunction. Our results show that MG reduces serine-1177 phosphorylation of eNOS in parallel with high glucose, an effect prevented by the MG scavenger aminoguanidine, implicating MG as a possible mediator of the effect of high glucose on reduced eNOS phosphorylation and activity.

So how does oxidative stress fit with the data presented in our study? NADPH oxidase is a key enzyme responsible for overproduction of superoxide anion and an increase in oxidative stress in endothelial dysfunction (Gao and Mann, 2009). We found that both MG and high glucose increased NADPH oxidase activity and ROS production that was prevented by the NADPH oxidase inhibitor, apocynin, and the MG-scavenging antioxidant, NAC, respectively, in RAECs and HUVECs. MG has been shown to increase NADPH oxidase activity and superoxide production in other cell types such as vascular smooth muscle cells (Chang *et al.*, 2005) and neutrophils (Ward and McLeish, 2004).

Are the effects of MG or high glucose on eNOS activity and NO production direct or through an increase in oxidative stress? Our results with eNOS activity assay (Fig. 7-6A), apocynin and NAC indicate the effects to be partly direct on the eNOS enzyme itself. Inhibiting superoxide with apocynin did not completely restore the ACh-induced relaxation of aortic rings (Fig. 7-1E, F) or BK-stimulated NO production in HUVECs (data not shown) that

was attenuated by MG or high glucose. On the contrary NAC, which can also scavenge MG (Vasdev *et al.*, 1998), restored ACh-induced relaxation of rings (Figs. 7-1C, D) and BK-stimulated NO production and cGMP increase in RAECs and HUVECs (Figs. 7-3, 7-4 and 7-5) that was attenuated by MG and high glucose.

MG and high glucose also significantly reduced GSH levels and expression of GSH-reductase, which was prevented by aminoguanidine, in HUVECs (Fig. 7-8) and RAECs (data not shown). GSH plays a key role in the degradation of MG by the glyoxalase enzymes (Dakin and Dudley, 1913). A reduction in GSH levels would delay the degradation of MG. Glutathione reductase replenishes GSH by reducing oxidized glutathione (GSSG) and is an antioxidant enzyme (Zhao *et al.*, 2009). MG has been shown to reduce GSH and GSH reductase levels in VSMCs (Wu and Juurlink, 2002). It should be pointed out besides MG, other reactive aldehydes, *viz*. glyoxal and 3-deoxyglucosone are formed from degradation of glucose (Thornalley *et al.*, 1999). According to one report 2.7 fold more 3-deoxyglucosone and 21 fold more glyoxal was formed from glucose in phosphate buffer (Thornalley *et al.*, 1999). However, of the three aldehydes MG is the most reactive and widely studied as an AGEs precursor.

Thus, our data provides a possible link between hyperglycemia and endothelial dysfunction with MG as the mediator of the endothelial dysfunction induced by high glucose. Therefore, MG is a potential target for preventive strategies against hyperglycemia-induced endothelial dysfunction and its sequelae. The potential of NAC needs to be researched in this regard. NAC is already clinically used in patients for conditions such as acetaminophen overdose, influenza viral infection, chronic obstructive pulmonary disease and pulmonary fibrosis (Millea, 2009). Aminoguanidine was found to be toxic in clinical trials as an AGEs

scavenger (Freedman *et al.*, 1999). However, for experimental studies aminoguanidine is the most effective and commonly used MG and AGEs scavenger (Desai and Wu, 2007, Lo *et al.*, 1994, Wang *et al.*, 2007b), and was a rational choice in our study as an MG scavenger.

In conclusion, hyperglycemia-induced endothelial dysfunction is receiving increasing attention as an early preventable event. High glucose-induced endothelial dysfunction is most likely mediated by MG. Development of specific and safe MG scavengers may prove very useful in blocking the multiple deleterious effects of hyperglycemia, including endothelial dysfunction and vascular complications of diabetes.

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Conflict of interest : None

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CHAPTER 8

Chronic methylglyoxal infusion by minipump causes pancreatic β cell dysfunction and induces type 2 diabetes in Sprague-Dawley rats

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Diabetes

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Abstract

The incidence of high dietary carbohydrate-induced type 2 diabetes mellitus is increasing worldwide. Methylglyoxal (MG) is a reactive glucose metabolite and a major precursor of advanced glycation end products (AGEs). MG levels are elevated in diabetic patients. It is not known if MG is a causative factor in the pathogenesis of diabetes. We investigated the effects of chronic administration of MG on glucose tolerance and β-cell insulin secreting mechanism in 12 week old male Sprague-Dawley rats. Synthesis of reduced glutathione (GSH), which plays a key role in MG degradation, was inhibited in one group of rats with buthionine-l-sulfoximine (BSO). MG was administered by continuous infusion with a subcutaneous mini-osmotic pump for 28 days. We measured MG and GSH levels by HPLC, performed *in vivo* glucose tolerance test, glucose uptake in adipose tissue, insulin secretion from isolated pancreatic islets, western blotting and mRNA, and apoptosis tests for islet cells. In rats treated with MG and MG+BSO, MG levels were significantly elevated in plasma, pancreas, adipose tissue and skeletal muscle, fasting plasma glucose was elevated, while insulin and GSH were reduced. These two groups also had impaired glucose tolerance, reduced insulin-stimulated glucose uptake and GLUT-4 expression in adipose tissue. Furthermore, in the pancreatic β-cells MG and MG+BSO reduced insulin secretion, PDX-1 MafA, and GLUT-2 mRNA or protein expression, increased cEBP/β protein or mRNA, and caused apoptosis. Alagebrium, an MG scavenger and an AGE-breaking compound, attenuated the effects of MG. Chronic MG induces biochemical and molecular abnormalities characteristic of type 2 diabetes and is a possible mediator of high carbohydrate-induced type 2 diabetes.

Key words: Methylglyoxal, insulin resistance, glucose intolerance, type 2 diabetes.

Introduction

Type 2 diabetes mellitus (T2DM) is characterized by hyperglycemia, insulin resistance, and progressive decrease in insulin secretion from the pancreas (Field, 1962). A genetic predisposition has been found in many patients. More recently there has been a staggering increasing in the incidence of T2DM, many of the cases being reported in children. This explosive increase is attributed to a diet high in carbohydrates, fat, and a sedentary lifestyle (Schwartz, 2008; van Dam *et al*., 2002; Weigensberg *et al*., 2009; Willett *et al*., 2002; Clark 2009). Oxidative stress is associated with diabetes mellitus and has been proposed as one of the causative factors of diabetes (Simmons, 2006; Shah *et al*., 2007). An increase in oxidative stress caused the insulin resistance of Zucker obese rats to progress to T2DM in one week (Laight *et al*., 1999).

Methylglyoxal (MG) is a reactive dicarbonyl metabolite of glucose, and to a much lesser extent of fatty acid, and protein metabolism (Desai and Wu, 2007). MG reacts with and modifies certain proteins to form advanced glycation end products (AGEs) (Desai and Wu, 2007; Vander Jagt, 2008). AGEs are implicated in the pathogenesis of vascular complications of diabetes (Vander Jagt, 2008; Vlassara *et al*., 1994). Plasma MG levels in healthy humans are 1 μM or less and are elevated 2-4 fold in diabetic patients with a positive correlation to the degree of hyperglycemia (Wang *et al*., 2007; McLellan *et al*., 1994). Under physiological conditions the highly efficient glyoxalase system degrades MG into D-lactate with the help of reduced glutathione (GSH) (10, 14) and keeps plasma MG levels at around 1 μM or less (Wang *et al*., 2007; McLellan *et al*., 1994). We have shown that incubation of vascular smooth muscle cells with 25 mM glucose or fructose for 3 h increases MG production 3.5 or 3.9 fold, respectively, and increases oxidative stress (Dhar *et al*., 2008). *In vitro* incubation of MG with

insulin modifies the structure of the insulin molecule, in a way that impairs insulin-mediated glucose uptake in adipocytes (Jia *et al*., 2006). Incubation of cultured L6 muscle cells with high concentrations of MG (2.5 mM) for 30 min impaired insulin signaling (Riboulet-Chavey *et al*., 2006). However, the *in vitro* studies cannot establish whether MG is the cause of diabetes or an effect of diabetes.

The molecular mechanisms of high dietary carbohydrate induced T2DM are not entirely clear. It is possible that high carbohydrate-induced chronic elevation of MG causes cumulative pathologic changes that contribute to the development of insulin resistance and T2DM. We have recently shown that a single acute dose of MG (50 mg/kg i.v.) administered to 12 week old male Sprague-Dawley (SD) rats causes insulin resistance and reduced adipose tissue insulin-stimulated glucose uptake (Dhar *et al*., 2010). Here we report the results of a comprehensive study on the effects of chronically administered MG on *in vivo* glucose tolerance, adipose tissue glucose uptake and insulin secretion from isolated pancreatic islets, and the underlying molecular mechanisms. We administered MG by continuous infusion *via* an osmotic minipump for 28 days, a method used for the first time to administer MG, which is expected to closely mimic the supposedly continuous production of MG in the body and avoid the excessive peaks in plasma concentrations of MG that can result from daily intraperitoneal administration.

METHODS

Animals

All animal protocols were approved by the Animal Care Committee of the University

of Saskatchewan. Male Sprague Dawley (SD) rats, 12 weeks old, from Charles River Laboratories, Quebec, Canada, were treated according to guidelines of the Canadian Council on Animal Care.

The rats were from the same batch and similar in weight. MG (40% solution) was administered to 12 week old male SD rats for 28 days by means of an osmotically driven infusion minipump (Alzet® 2ML4, Durect Corporation, Cupertino, CA, USA) implanted subcutaneously on the back following procedure provided on a video by the company. This pump holds a fixed volume (2 ml) of drug and releases a continuous small amount of MG into the body at a rate of 2.5 µl/h or 60 µl/day, amounting to 60 mg/kg/day. Control rats were administered 0.9% saline (2.5 µl/h) by means of subcutaneously implanted pump. GSH is an antioxidant, which also plays a key role in degrading MG. Since oxidative stress has been implicated as a possible pathogenetic factor for T2DM (Simmons 2006; Shah *et al*., 2007) we also treated rats with buthionine-l-sulfoximine (BSO), an inhibitor of glutamyl cysteine synthetase (Meister, 1983), which prevents GSH synthesis, decreases MG degradation, and increases oxidative stress. Alagebrium is a MG scavenger and an AGE-breaking compound (Dhar *et al*., 2010; Wolffenbuttel, 1998).

After one week of acclimatization the rats were divided into the following treatment groups (n = 8 each): **1. Control** – 0.9% saline, **2. MG** (60 mg/kg/day), **3. MG** (60 mg/kg/day) + **alagebrium** (ALA, 30 mg/kg/day in drinking water), **4. ALA** alone (30 mg/kg/day in drinking water), **5. MG** (60 mg/kg/day) + **BSO** (30 mg/kg/day in drinking water), **6. BSO** alone (30 mg/kg/day in drinking water). All the treatments were for 28 days.

Biochemical parameters

Blood was collected from anesthetized rats from different treatment groups, the plasma was separated and analyzed for total cholesterol, triglycerides, high density lipoprotein (HDL), creatine kinase (CK), creatinine, alanine transaminase (ALT) and aspartate aminotransferase (AST).

Oral Glucose Tolerance Test (OGTT)

After overnight fasting, an oral glucose tolerance test (GTT) was performed. Briefly, the trachea, left jugular vein, and right carotid artery were cannulated in anesthetized rats. After collecting a basal blood sample an oral glucose load (1 g/kg) was given with a stomach tube. Further blood samples were collected at 5, 15, 30, 60 and 120 min from the carotid artery. Plasma glucose levels were determined using a glucose assay kit (BioAssay Systems, Hayward, CA, USA) and insulin levels were measured with a rat insulin ELISA assay kit (Mercodia Inc., Winston Salem, NC, USA).

Insulin release from freshly isolated pancreatic islets

The pancreatic islets were freshly isolated from SD rats as described previously (Wu *et al*., 2009). Briefly, rats were anesthetized with isoflurane, the pancreatic duct was cannulated and injected with ~ 7 ml of ice-cold collagenase (Type IV, 5 mg/ml, Worthington Biochemical Corporation, Lakewood, NJ, USA) solution in Krebs buffer. The pancreas was cut out, washed in Krebs buffer and finely chopped into small pieces with Mcllwain tissue chopper. The tissue was digested with 20 ml collagenase (Type IV) for 20 min at 37 °C in a shaker bath and then stopped by addition of 20 ml of calcium-free ice-cold Krebs buffer. The digested tissue was then centrifuged at 1000 rpm for 10 min and washed twice with Krebs buffer and thereafter

transferred into a petri dish. Islets were separated under a dissection microscope. The identity of islets was confirmed under a high power microscope. For each treatment 10-20 islets were used in eppendorf tubes. The islets were incubated for 30 min at 37°C in Krebs buffer containing either 5 mM or 20 mM glucose. The incubation medium was collected and analyzed for released insulin with insulin assay kit

Glucose uptake

Adipose tissue was isolated and washed in Dulbecco's modified Eagle medium (DMEM) without glucose and chopped. The chopped tissue was transferred into eppendorf tubes, weighed and incubated in DMEM containing collagenase solution (1.5 mg/ml) for 30 min. Insulin was added at a final concentration of 100 nM and incubated for another 30 min. Finally, after the addition of [^{3}H]-2-DOG (0.1 µCi/500 µl) and glucose (50 µM) to the medium, the tissue was incubated for another 20 min. All incubations were performed at 37°C. The incubation was stopped by washing three times with ice-cold phosphate buffered saline. The cells were lysed in 0.1% sodium dodecyl sulfate (SDS) and 1 N NaOH and then transferred into scintillation vials for counting (Beckman LS 3801 scintillation counter).

MG Assay

MG was measured by a specific and sensitive HPLC method (Dhar *et al*., 2009). Briefly, MG was derivatized with *o*-phenylenediamine (*o*-PD) to form the quinoxaline product, 2-methylquinoxaline. The samples were incubated on dark for 24 h with 0.4 N perchloric acid (PCA) and 10 mM *o*-PD at room temperature. Samples were centrifuged at 12000 rpm for 10 min. The 2-methylquinoxaline and 5-methylquinoxaline, which was added

to the samples as the internal standard, were quantified on a Hitachi D-7000 HPLC system (Hitachi, Ltd., Mississauga, ON, Canada) *via* Nova-Pak® C18 column (3.9×150 mm, and 4 µm particle diameter, Waters Corporation, Milford, MA, USA).

Measurement of reduced glutathione levels

The GSH levels in the plasma and organs were determined by derivation with 5, 50-dithio-bis (2-nitrobenzoicacid), and reverse-phase HPLC using ultra-violet Detection, as described previously (Kamenic *et al*., 2000).

Determination of cell apoptosis:

(i) Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The TUNEL assay was done using TUNEL assay kit (Roche Diagnostics, Indianapolis, IN, USA). In brief, paraformaldehyde- fixed and optimal cutting temperature (OCT) compound-embedded sections of pancreas were cut into 4µM on glass slides. The sections were washed twice with PBS. After permeation with 0.1% Triton X-100 for 5 min and two washes with PBS, the tissue sections were incubated with blocking solution (3% H_2O_2 in methanol) for 10 min. After washing with PBS the slides were incubated with TUNEL reagent for 1h in dark and then converter POD for 30 min. After rinsing with PBS, the DAB reagent was added, the sections were mounted under glass cover slips and analyzed under light microscope

DNA was isolated from pancreas using DNA extraction kit (R & D Systems, Minneapolis, MN, USA). Equal amount and concentration (1 µg) of DNA was loaded on 1% agarose gel and run at 100V for 2h. The gel was visualized on Syngene bio-imaging system

(Syngene, Frederick, MD, USA).

Isolation of plasma membrane for GLUT4

Plasma membrane from adipose tissue was isolated using plasma membrane isolation kit (BioVision Inc., MountainView, CA, USA). Briefly, adipose tissue was homogenized in 2-3 volume of the 1x homogenization buffer, until completely lysed (30-50 times). The sample was centrifuged at 700 g for 10 min at 4°C, the supernatant was collected and centrifuged at 10,000 g for 30 min at 4°C. The supernatant is the cytosol fraction and the pellet is the total cellular membrane protein (containing proteins from both plasma membrane and cellular organelle membrane). The total membrane protein pellet was resuspended in 200 µl of the upper phase solution and an equal volume of lower phase solution was added, mixed well and incubated on ice for 5 min. The sample was then centrifuged at 3500 rpm for 5 min. The upper phase was carefully transferred to a new tube and extracted by adding 100 µl of the lower phase solution. The upper phase was diluted in five volumes of water, kept on ice for 5 min and spun at top speed for 10 min at 4°C. The supernatant was removed and the pellet containing the plasma membrane protein was used for membrane GLUT4 determination.

Western immunobloting

Isolated pancreas and adipose tissue were homogenized using polytron homogenizer in a homogenization buffer. The supernatants were resolved on 10% SDS-PAGE gel and transferred to PVDF membrane. The membranes were blocked with 5% non-fat dry milk solution for 1h and incubated overnight with primary antibodies for GLUT4 (plasma membrane and total), GLUT2, PDX-1, Maf-A, C/EBPβ, β-actin (Santa Cruz Biotechnology

Inc., Santa Cruz, CA, USA) and then with horse radish peroxidase conjugated secondary antibody for 1h. The reactions were visualized using ECL reagent and exposed to X-ray film (Kodak scientific imaging film, Eastman Kodak Company, Rochester, NY, USA).

Real time Quantitative PCR (RT-PCR)

RNA was isolated from the pancreas using RNA isolation kit (Qiagen sciences, Germantown, MD, USA). The total RNA was reverse-transcribed in triplicate using RevertAid™ H Minus M-MuLV reverse transcriptase (MBI, Fermentas Burlington, ON, Canada) in the presence of 5x RT buffer (MBI, Fermentas), Random primer (Invitrogen Corporation, Carlsbad, CA, USA), dNTP mixture (Amersham, Pittsburgh, PA, USA) at 42°C for 50 min, followed by 72°C for 10 min. The pre-designed primers for PDX-1 and Maf-A were from Qiagen Sciences (Germantown, MD, USA). The real-time PCR was carried out in an iCycler iQ apparatus (Bio-Rad, Life Science Research, Hercules, CA, USA) associated with the ICYCLER OPTICAL SYSTEM software (version 3.1) using SYBR Green PCR Master Mix (Bio-Rad, Life Science Research, Hercules, CA, USA). All PCRs were triplicated and performed in 96-well optical-grade PCR plates and run for 45 cycles at 95°C for 20 s, 62°C for 1 min, and 72°C for 30 s. After cycling, melting curves of the PCR products were acquired by stepwise increase of the temperature from 62° to 95°C.

Chemicals and Statistical analysis

All chemicals were of analytical grade. Methylglyoxal and *o*-phenylenediamine (*o*-PD) were purchased from Sigma Aldrich, Oakville, ON, Canada. Alagebrium (formerly known as ALT-711) was a generous gift from Synvista Therapeutics, Inc. (Montvale, NJ,

USA). Data are expressed as mean ± SEM and analyzed using one way ANOVA and *post hoc* Bonferroni's test. *P* value less than 0.05 was considered significant.

RESULTS

Chronic methylglyoxal treatment significantly alters metabolic characteristics of SD rats

MG, MG+BSO and BSO treatment for 4 weeks significantly increased fasting plasma glucose (Fig. 8-2A inset), total cholesterol and triglycerides (Table 8-1), and decreased fasting plasma insulin (Fig. 8-2B inset) and high density lipoprotein (HDL) (Table 8-1) levels as compared to the control group. There was no significant difference in the body weight between the treatment groups (Table 8-1), and markers indicating tissue or organ damage such as serum creatine kinase (CK) for muscle damage, creatinine for kidney function, alanine transaminase (ALT) and aspartate aminotransferase (AST) for liver damage (Table 8-1). Pretreatment with ALA (30 mg/kg/day) significantly attenuated MG-induced changes in the metabolic parameters (Fig. 8-2 insets, Table 8-1).

Methylglyoxal and GSH levels were different among treatment groups

MG and MG+BSO treated groups had significantly elevated plasma, pancreatic, adipose tissue and skeletal muscle MG levels compared to control (Fig. 8-1A, B) that were attenuated by pretreatment with ALA in the MG+ALA group. BSO alone also significantly elevated plasma, pancreatic, adipose tissue and skeletal muscle MG compared to control (Fig. 8-1A, B). MG levels were higher in plasma and tissues in the MG+BSO group compared to MG and BSO alone groups (Fig. 8-1A, B). MG and MG+BSO treatment significantly reduced GSH levels in the plasma, pancreas and skeletal muscle (Fig. 8-1C, D). ALA pretreatment

attenuated the decrease in plasma GSH induced by MG (Fig. 8-1C, D). BSO alone also reduced plasma, skeletal muscle and pancreatic GSH compared to control (Fig. 8-1C, D). Adipose tissue and pancreas had much lower GSH levels compared to skeletal muscle (Fig. 8-1D).

Chronic methylglyoxal impaired glucose tolerance in SD rats

MG and MG+BSO treatment significantly impaired *in vivo* glucose tolerance determined after an oral glucose load in SD rats (Fig. 8-2A). Plasma glucose levels were significantly higher in the MG and MG+BSO group compared to control even 2 h after the glucose load. ALA pretreatment attenuated the impaired glucose tolerance induced by MG (Fig. 8-2A).

MG and MG+BSO treated groups had significantly lower plasma insulin levels compared to the control group in the oral GTT (Fig. 8-2B). The plasma insulin levels were lower in the MG+BSO group compared to the MG alone group. The plasma insulin levels were significantly lower than control even 2 h after the glucose load in the MG and MG+BSO groups. ALA pretreatment attenuated the reduced insulin levels induced by chronic MG treatment (Fig. 8-2B).

Chronic methylglyoxal treatment reduces glucose uptake and plasma membrane GLUT4 expression in adipose tissue

Insulin-stimulated glucose uptake was evaluated in adipose tissue freshly isolated from rats after different treatments for 28 days. There was a significant decrease in insulin-stimulated glucose uptake in rats treated with MG or MG+BSO compared to those from

control (Fig. 8-3A). ALA attenuated the reduced insulin-stimulated glucose uptake by MG. To understand the possible mechanism of MG induced impaired glucose tolerance and reduced glucose uptake, the adipose tissue GLUT4 translocation to the plasma membrane was determined. There was a significant decrease in plasma membrane GLUT4 in MG and MG+BSO treated rats (Fig. 8-3B). ALA attenuated the reduced plasma membrane GLUT4 induced by MG. BSO alone also reduced plasma membrane GLUT4 (Fig. 8-3B).

Chronic methylglyoxal treatment reduces total insulin content and glucose-stimulated insulin release from pancreas

The pancreatic insulin content was significantly reduced in MG and MG+BSO treated groups compared to control (Fig. 8-4). ALA attenuated the reduction in insulin content by MG (Fig. 8-4). We further investigated whether MG inhibits pancreatic insulin release. Basal and glucose-stimulated insulin release from isolated pancreatic islets was significantly reduced in MG and MG+BSO groups compared to control (Fig. 8-5A). BSO alone also significantly reduced glucose-stimulated insulin secretion compared to control but less than MG+BSO group (Fig. 8-5A). ALA significantly attenuated MG-induced decrease in glucose-stimulated insulin release from the pancreatic islets (Fig. 8-5A).

Effects of chronic MG on insulin synthesis/secretion pathway in pancreas

To determine the cause of MG-induced reduction of basal plasma level and glucose-stimulated insulin release from pancreatic islets we looked at the molecular mechanisms of insulin synthesis and secreting pathways. GLUT2 is the transporter for glucose entry into islet cells. PDX-1, Maf-A are negative regulators whereas C/EBPβ is a positive regulator of insulin

gene transcription. There was a significant decrease in GLUT2 (Fig. 8-5B), PDX-1 and Maf-A protein expression in MG and MG+BSO treated rats [Fig. 8-6A(i), A(ii)). There was also a significant decrease in mRNA expression of PDX-1 and Maf-A in MG and MG+BSO treated rats as compared to control [Fig. 8-6B(i), B(ii)]. ALA attenuated the decrease in GLUT2 (Fig. 8-5B), PDX-1 and Maf-A protein and mRNA induced by MG [Fig. 8-6A(i), A(ii), 8-6B(i), B(ii)]. At the same time there was a significant increase in C/EBPβ protein expression and mRNA in MG and MG+BSO treated rats [Fig. 8-6A(iii), B(iii)]. MG-induced increase in C/EBPβ protein and mRNA was prevented by ALA [Fig. 8-6A(iii), 8-6B(iii)].

Chronic methylglyoxal treatment induces apoptosis of pancreatic β-cells

After chronic MG and MG+BSO administration, there was significant DNA fragmentation (Fig. 8-7A) and significant positive BrdUTP staining indicating apoptosis (Fig. 8-7Bii, iii) of pancreatic β cells as compared to control. ALA significantly attenuated MG induced apoptosis and DNA fragmentation in chronic MG treated rats (Fig. 8-7A, Biv). BSO alone also partially induced apoptosis (Fig. 8-7Bv) but had no effect on DNA fragmentation (Fig. 8-7A).

Table 8-1

Plasma levels of different substances in Sprague-Dawley rats treated with methylglyoxal (MG). 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic pump for 28 days to all groups of rats (*n*=6 each). The MG scavenger, alagerbium (ALA, 15 mg/kg/day in drinking water) (MG+ALA) or glutathione synthesis inhibitor, buthionine-L-sulfoximine (BSO, 30 mg/kg/day in drinking water) (MG+BSO and BSO) were administered to some groups of rats. The control group received only saline (0.9% by pump). After 28 days basal plasma levels of substances listed in the table were measured.

Parameter	Control	MG	MG+BSO	MG+ALA	BSO
Body weight (g)	558 ± 18	556 ± 9	582 ± 13	588 ± 7	536 ± 33
Total Cholesterol	1.8±0.1	2.4±0.05***	2.8±0.05***††	1.9±0.03††	2.1±0.1**‡‡‡
HDL	1.2±0.05	0.7±0.06**	0.46±0.05***†	1.0±0.07†	0.78±0.1**‡
Triglycerides	0.2±0.03	0.5±0.04***	0.7±0.02***††	0.3±0.02††	0.4±0.05**‡‡‡
Creatinine	55±5	66±6	75±8	52±2	58±5
CK	315±20	365±25	378±23	332±15	355±21
ALT	60±10	65±9	71±10	59±8	63±7
AST	46±5	50±8	56±9	45±6	48±4

Abbreviations: HDL, high density lipoprotein; CK, creatinine kinase; AST, aspartate aminotransferase; ALT, Alanine transaminase

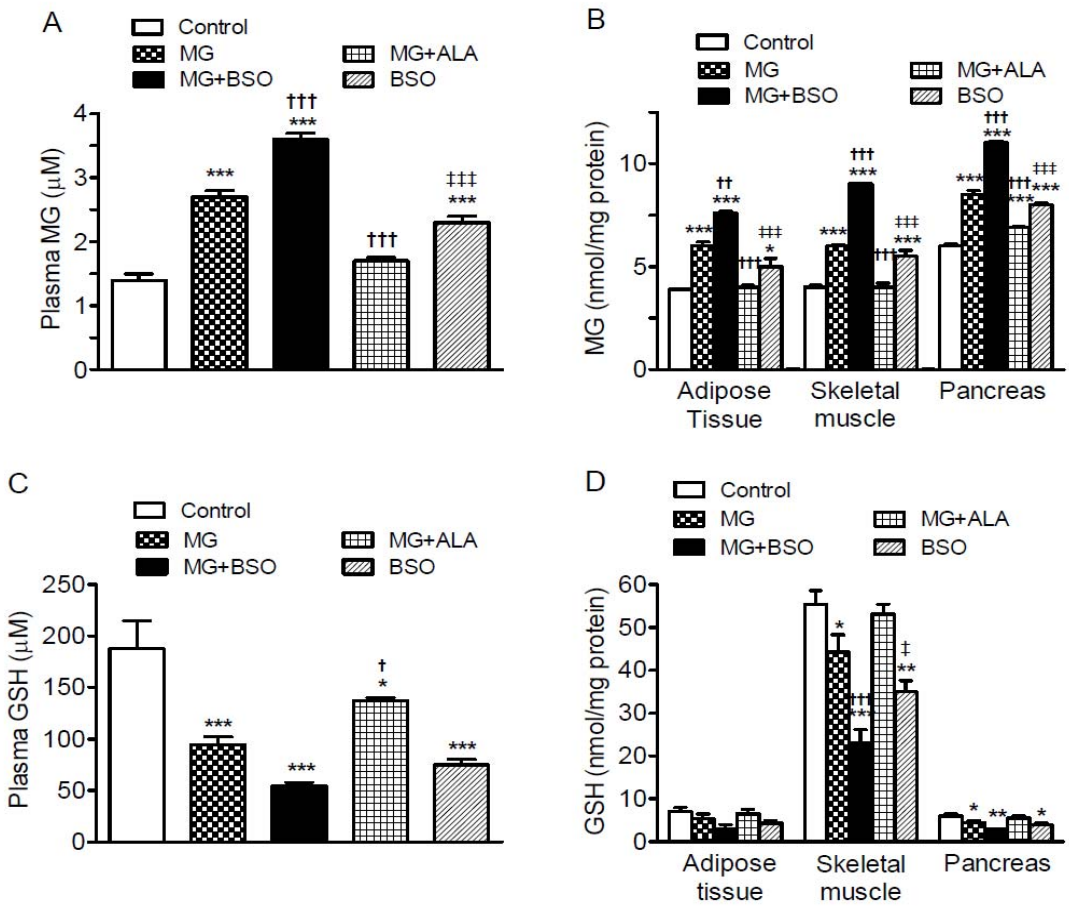


Fig. 8-1 Methylglyoxal (MG) levels are elevated and reduced glutathione (GSH) levels are decreased in Sprague-Dawley rats chronically treated with MG. 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic minipump for 28 days to all groups of rats (n=6 each). The MG scavenger, alagerbium (ALA, 30 mg/kg/day in drinking water) (MG+ALA) or glutathione synthesis inhibitor, buthionine-L-sulfoximine (BSO, 30 mg/kg/day in drinking water) (MG+BSO and BSO) were administered to some groups of rats. The control group received only saline (0.9% by pump). After 28 days MG and GSH levels were determined by HPLC in (A, C) plasma and (B, D) organs. *P<0.05, **P<0.01, ***P<0.001 *vs* respective control, $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01, $^{\dagger\dagger\dagger}P$< 0.001 *vs* respective MG group, $^{\ddagger}P$<0.05, $^{\ddagger\ddagger}P$<0.01, $^{\ddagger\ddagger\ddagger}P$<0.001 *vs*. respective MG+BSO group.

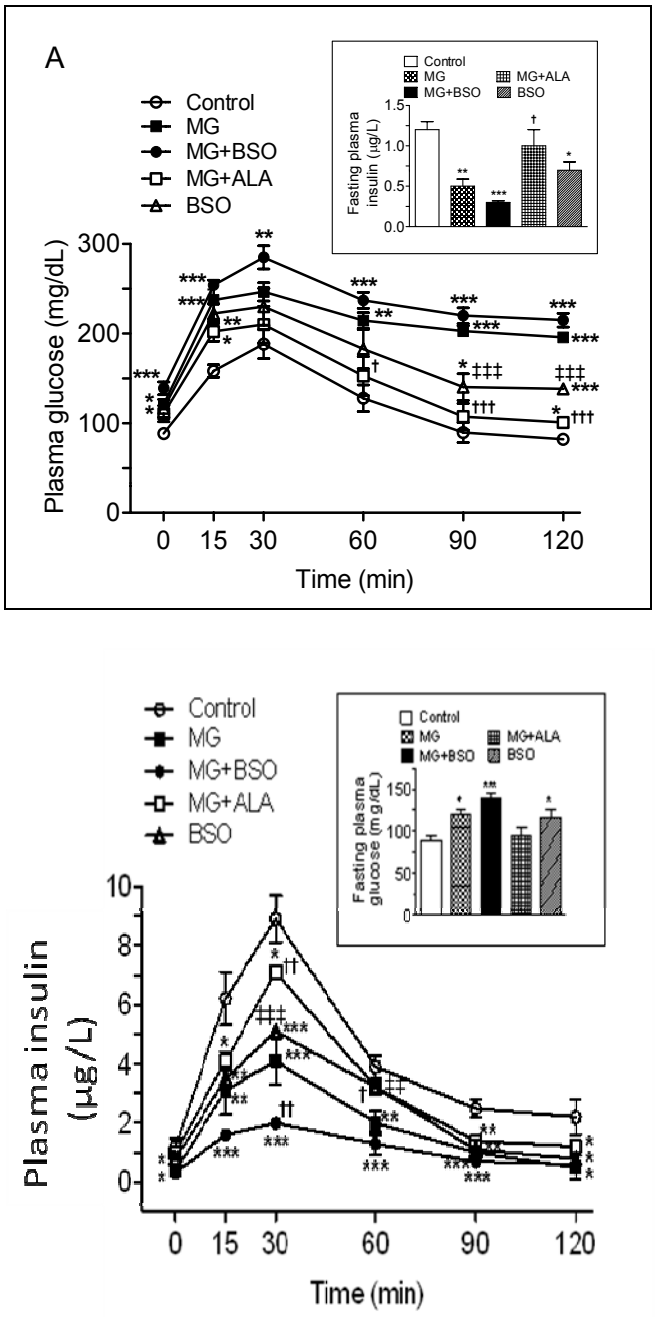


Fig. 8-2 Fasting plasma glucose is elevated, insulin is reduced and oral glucose tolerance test is impaired in Sprague-Dawley rats chronically treated with methylglyoxal (MG). 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic minipump for 28 days to all groups of rats (*n*=6 each). The MG scavenger, alagerbium (ALA,

30 mg/kg/day in drinking water) (MG+ALA) or glutathione synthesis inhibitor, buthionine-L-sulfoximine (BSO, 30 mg/kg/day in drinking water) (MG+BSO and BSO) were administered to some groups of rats. The control group received only saline (0.9% by pump). After 28 days the rats were fasted overnight, anesthetized and cannulated. A basal zero min blood sample was taken from the carotid artery. After that an oral glucose load (1 g/kg body wt) was administered and blood samples were collected from the carotid artery at different times up to 120 min. Plasma was separated and analyzed for (A inset) basal glucose, (B inset) basal insulin, and (A) glucose and (B) insulin levels following the glucose load. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs* respective control at the same time point, $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ *vs* respective MG group at the same time point, $^{\ddagger\ddagger}P<0.01$, $^{\ddagger\ddagger\ddagger}P<0.001$ *vs*. respective MG+BSO group at the same time point.

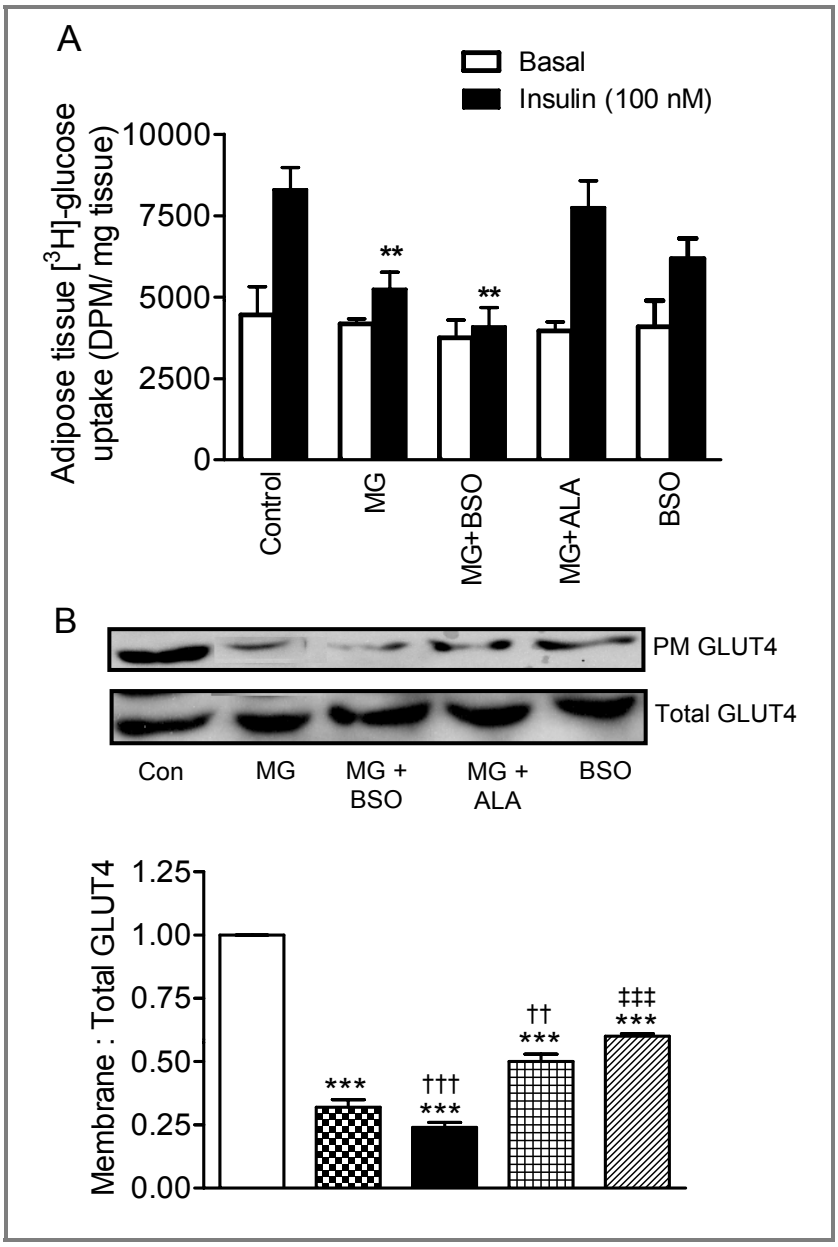


Fig. 8-3 Adipose tissue glucose uptake and plasma membrane GLUT4 protein are reduced in chronic methylglyoxal (MG) treated Sprague-Dawley rats. 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic minipump for 28 days to all groups of rats (*n*=6 each). The MG scavenger, alagerbium (ALA, 30 mg/kg/day in drinking water) (MG+ALA) or glutathione synthesis inhibitor, buthionine-L-sulfoximine (BSO, 30

mg/kg/day in drinking water) (MG+BSO and BSO) were administered to some groups of rats. The control group received only saline (0.9% by pump). After 28 days the rats were fasted overnight, and (A) abdominal visceral adipose tissue was removed and tested for insulin-stimulated glucose uptake *in vitro*. (B) The adipose tissue was also subjected to Western blotting to determine the plasma membrane and total GLUT4 protein as described in methods. $*P<0.05$, $**P<0.01$ *vs* control, $^{\dagger}P<0.05$ *vs* MG group.

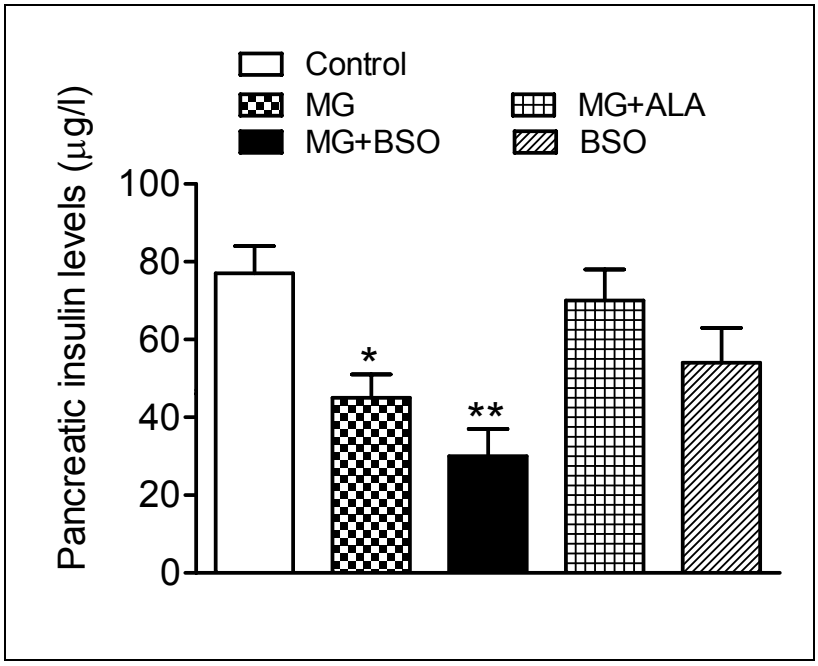


Fig. 8-4 Pancreatic insulin content is reduced in Sprague-Dawley rats chronically treated with methylglyoxal (MG). 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic minipump for 28 days to all groups of rats (n=6 each). The MG scavenger, alagerbium (ALA, 30 mg/kg/day in drinking water) (MG+ALA) or glutathione synthesis inhibitor, buthionine-L-sulfoximine (BSO, 30 mg/kg/day in drinking water) (MG+BSO and BSO) were administered to some groups of rats. The control group received only saline (0.9% by pump). After 28 days the rats were fasted overnight, and the pancreas was removed and evaluated for insulin content. *P<0.05, **P<0.01 *vs* respective control.

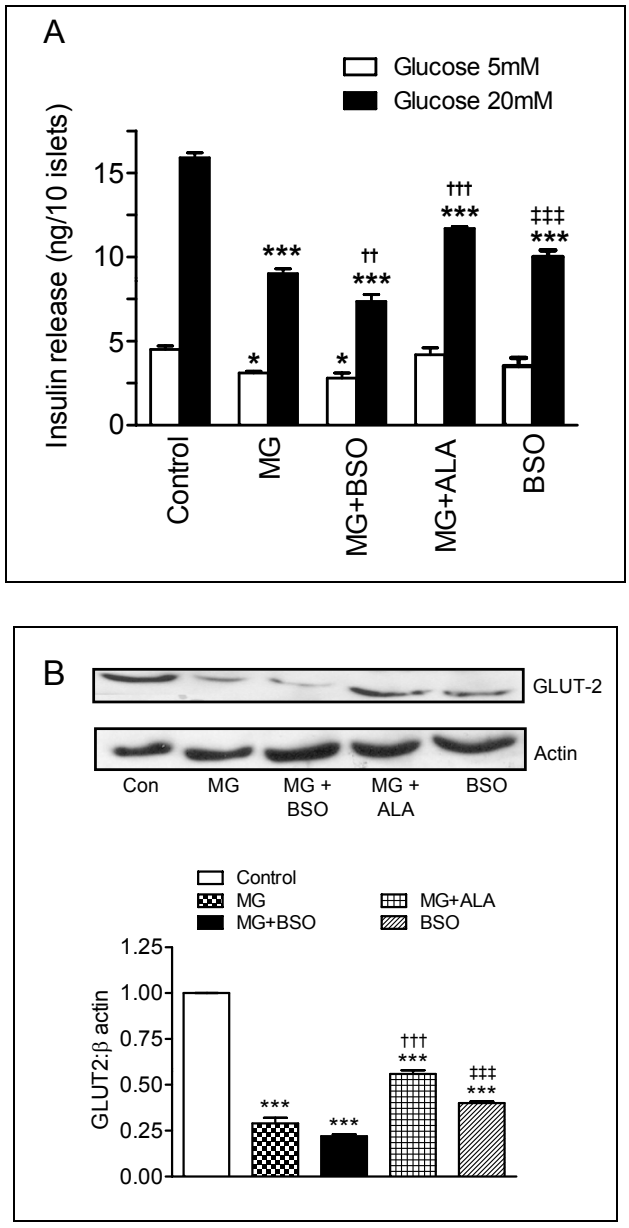


Fig. 8-5 Pancreatic GLUT2 and insulin release from isolated pancreatic islets are reduced in Sprague-Dawley rats chronically treated with methylglyoxal (MG). 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic minipump for 28 days to all groups of rats (*n*=6 each). The MG scavenger, alagerbium (ALA, 30 mg/kg/day in drinking water) (MG+ALA) or glutathione synthesis inhibitor, buthionine-L-sulfoximine (BSO, 30 mg/kg/day in drinking water) (MG+BSO and BSO) were administered to some

groups of rats. The control group received only saline (0.9% by pump). After 28 days the rats were fasted overnight, and (A) the pancreatic islets were isolated as described in methods. Glucose-stimulated insulin secretion was evaluated in the isolated islets *in vitro*. (B) The pancreatic tissue was removed and processed for determination of GLUT2 protein expression by western blotting. **P*<0.05, ****P*<0.001 *vs* respective control, $^{\dagger\dagger}P$<0.01, $^{\dagger\dagger\dagger}P$< 0.001 *vs* respective MG group, $^{\ddagger\ddagger\ddagger}P$<0.001 *vs*. respective MG+BSO group.

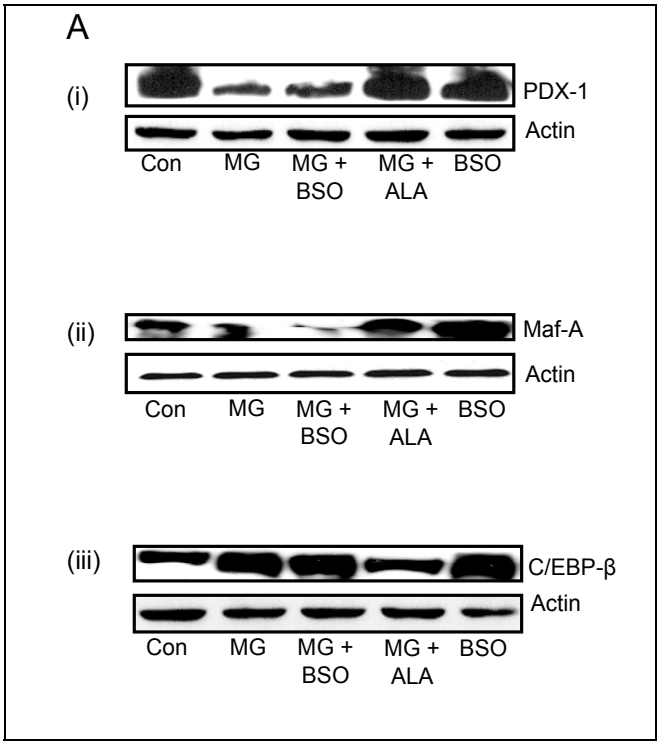


Fig. 8-6 (Next page contd.)

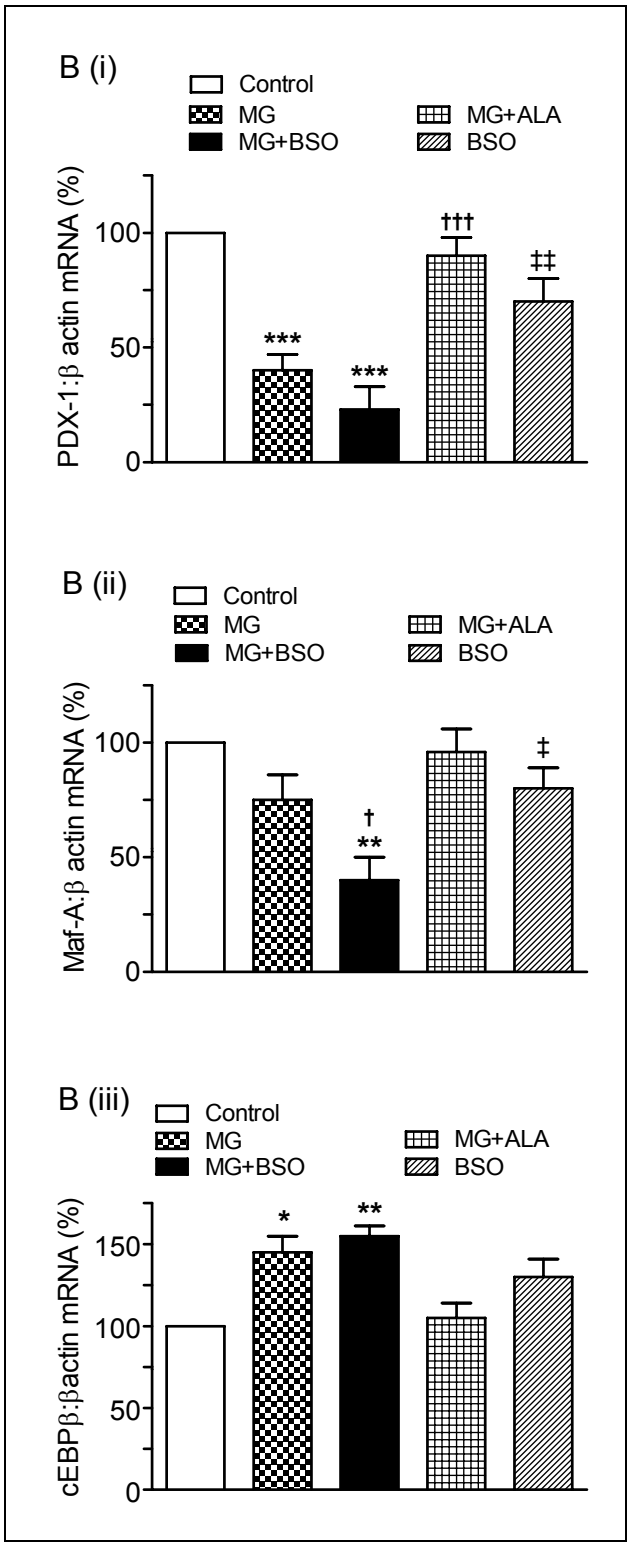
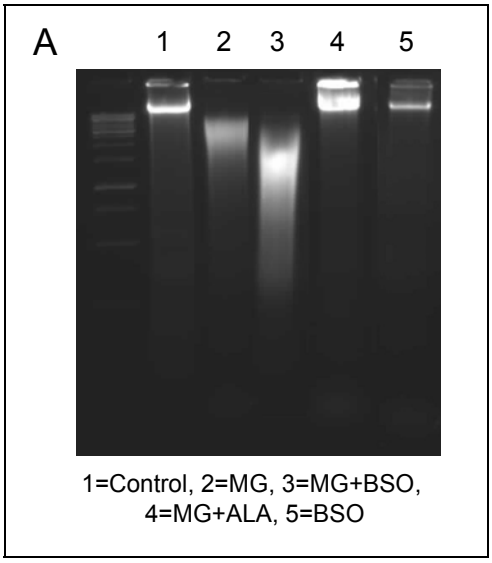


Fig. 8-6 Insulin gene transcription factors, pancreatic duodenal homeodomain-1 (PDX-1) and Maf-A protein expression and mRNA are reduced; and CCAAT/Enhancer Binding

Protein β (C/EBPβ) protein expression and mRNA are enhanced in Sprague-Dawley rats chronically treated with methylglyoxal (MG). 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic minipump for 28 days to all groups of rats (*n*=6 each). The MG scavenger, alagerbium (ALA, 30 mg/kg/day in drinking water) (MG+ALA) or glutathione synthesis inhibitor, buthionine-L-sulfoximine (BSO, 30 mg/kg/day in drinking water) (MG+BSO and BSO) were administered to some groups of rats. The control group received only saline (0.9% by pump). After 28 days the pancreatic tissue was removed and processed for determination of (Ai) PDX-1, (Aii) Maf-A and (Aiii) C/EBPβ protein expression by western blotting. (B) (i) PDX-1, (ii) Maf-A and (iii) C/EBPβ mRNA were also determined by RT-PCR in pancreatic tissue isolated from groups of rats described above. **P*<0.05, ***P*<0.01, ****P*<0.001 *vs* respective control, $^{\dagger}P$<0.05, $^{\dagger\dagger\dagger}P$< 0.001 *vs* respective MG group, $^{\ddagger}P$<0.05, $^{\ddagger\ddagger}P$<0.01 *vs*. respective MG+BSO group.



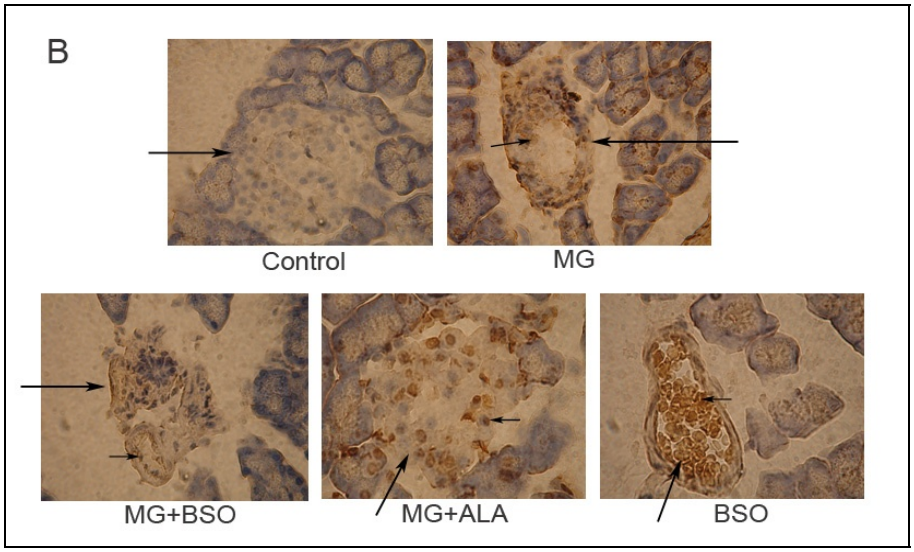


Fig. 8-7 Chronic methylglyoxal (MG) induces apoptosis in pancreatic islets in Sprague-Dawley rats. 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic minipump for 28 days to all groups of rats (*n*=6 each). The MG scavenger, alagerbium (ALA, 30 mg/kg/day in drinking water) (MG+ALA) or glutathione synthesis inhibitor, buthionine-L-sulfoximine (BSO, 30 mg/kg/day in drinking

water) (MG+BSO and BSO) were administered to some groups of rats. The control group received only saline (0.9% by pump). After 28 days (A) DNA was isolated and run on agarose gel to evaluate DNA integrity. (B) a part of the pancreas was removed and fixed in 4% paraformaldehyde. The tissue was processed for sectioning and stained for the terminal deoxynucleotidyl gtransferase dUTP nick end labeling (TUNEL) assay. Big black arrows show the pancreatic islet. Small black arrows show brown staining of apoptotic cells.

DISCUSSION

In the present study we report for the first time that MG administered by continuous infusion with a minipump for 28 days to Sprague-Dawley rats induces metabolic, biochemical and molecular abnormalities characteristic of type 2 diabetes mellitus. The most notable change induced by MG was an elevation of fasting plasma glucose and reduction of insulin level accompanied by a significantly impaired oral glucose tolerance test (Fig 8-1, 8-2). The oral GTT revealed abnormally elevated plasma glucose levels and reduced insulin levels. An investigation of the mechanisms of elevated plasma glucose revealed reduced insulin-stimulated glucose uptake in adipose tissue and plasma membrane GLUT-4 protein. At the same time the pancreatic islet β-cell insulin secreting machinery was significantly impaired in MG and MG+BSO treated rats. Glucose-stimulated insulin secretion was reduced in freshly isolated pancreatic islets, GLUT2 protein level was decreased, pancreatic insulin content was lower, protein as well as mRNA expression of insulin promoter transcriptional factors PDX-1 and Maf-A were significantly down-regulated whereas protein expression of C/EBPβ, a repressor of insulin gene transcription was significantly up-regulated in MG and MG+BSO treated rats. The deleterious effects of MG were attenuated by the MG scavenger and AGEs breaking compound, alagebrium (Dhar *et al*., 2010; Wolffenbuttel, 1998).

To the best of our knowledge, MG has not been previously administered chronically by continuous infusion. This is important because continuous infusion mimics the supposedly continuous production of MG in the body and it avoids the excessive peaks in plasma that are associated with periodic intraperitoneal or subcutaneous injections, which are necessary in a chronic study. The plasma levels of MG were significantly elevated between 2 and 4 fold in the MG and MG+BSO groups compared to control (Fig. 8-1A), similar to a range reported in

diabetic animals and patients (Wang *et al*., 2007; McLellan *et al*., 1994; Jia *et al*., 2007). MG levels were also significantly higher in the pancreas, adipose tissue and skeletal muscle, which can explain the metabolic changes observed in these organs/tissues. GSH levels were reduced in the plasma, pancreas, adipose tissue and skeletal muscle. GSH plays a central role in the degradation of MG by binding MG and making it available for degradation by the glyoxalase enzymes (Desai and Wu, 2007; Meister, 1983; Desai and Wu, 2008). A reduction in GSH would decrease MG degradation and contribute to its elevated levels, thus setting up a vicious cycle. This was observed in groups of rats treated with the GSH synthesis inhibitor, BSO (Meister, 1983), which further increased MG levels in the MG+BSO group and aggravated the effects of chronic MG. MG itself has been shown to reduce GSH levels (Fig. 8-1C, D) (Dhar *et al*., 2009; Desai and Wu, 2008). Pancreatic β cells have been reported to contain low levels of GSH and antioxidant capacity compared to other tissues making it more susceptible to oxidative damage (Robertson and Harmon, 2006). Our study supports this view since we also found lower GSH levels in pancreas as compared to skeletal muscle (Fig. 8-1D).

The fasting elevated glucose and low insulin levels in the plasma (Fig. 8-2A, B) are features of T2DM (Field, 1962). In the early stages of insulin resistance plasma insulin levels are higher than normal, at a stage when the pancreas secretes more insulin to overcome high glucose levels. As the disease progresses, the pancreas fails to secrete enough insulin and the plasma insulin levels are less than normal with higher plasma glucose levels (Field, 1962; Pick *et al*., 1998). The oral GTT revealed significantly impaired glucose tolerance and reduced insulin-response to the glucose load. An impaired GTT is a feature of the prediabetic insulin resistance as well as T2DM. One reason for the elevated plasma glucose levels is reduced response of insulin sensitive tissues to insulin due to defects in one or more steps of the insulin

signaling pathway. *In vitro* studies have shown that incubation of cultured 3T3-L1 adipocytes with MG reduced glucose uptake, decreased insulin-induced insulin-receptor substrate-1 (IRS-1) tyrosine phosphorylation, and decreased the activity of phosphatidylinositol 3-kinase (PI3K) (Riboulet-Chavey *et al*., 2006; Jia *et al*., 2007). In a genetic model of diabetes such as the Zucker obese rat a defect of glucose transport in muscle has been reported (Friedman *et al*., 1991). We found that MG and MG+BSO reduced membrane GLUT4 in the adipose tissue. GLUT4 is the glucose transporter isoform that carries glucose into the adipose and skeletal muscle cells in response to insulin. Insulin receptor activation in these tissues results in GLUT4 translocation to the cell membrane. We have recently shown that a single dose of MG (50 mg/kg i.v.) given to SD rats causes reduced membrane GLUT4 and insulin receptor substrate-1 phosphorylation in adipose tissue (Dhar *et al*., 2010).

The main reason for the reduced fasting plasma insulin and the reduced insulin response to the glucose load could be due to altered function of the pancreatic islets induced by MG. We observed that the total pancreatic insulin content was reduced in rats treated with MG and MG+BSO (Fig. 8-4). This is likely due to reduced transcription of insulin synthesis as indicated by reduced levels of the insulin gene promoters PDX-1 and Maf-A induced by MG (Fig. 8-6Bi, ii). PDX-1 is an essential regulator of both pancreatic exocrine and endocrine cell development. Inactivation of PDX-1 in the β-cells has been shown to impair β-cell function and cause diabetes (Brissova *et al*., 2005; Leibowitz *et al*., 2001; Robertson and Harmon, 2006). Similarly, Maf-A activates insulin as well as glucagon gene expression in the pancreatic islet β and α cells, respectively (Harmon *et al*., 2005). The expression and/or activities of PDX-1 and MafA are reduced in beta-cells in diabetes, resulting in decreased insulin synthesis and secretion (Kaneto *et al*., 2008), which supports our findings of MG as a

likely causative factor in T2DM.

MG and MG+BSO up regulated C/EBPβ in pancreas (Fig. 8-6Biii), which can also be responsible for the reduced insulin levels in these rats because C/EBPβ is a repressor of insulin gene transcription in β-cells and in diabetes animal models. C/EBPβ deletion reduces adiposity, hepatic steatosis and diabetes in Lepr db/db mice (Schroeder-Gloeckler *et al*., 2007). We also tested basal and glucose-stimulated insulin secretion from freshly isolated pancreatic islets which showed reduced insulin secretion in rats treated with MG and MG+BSO. These results support our previous study which showed that acute administration of MG (50 mg/kg i.v.) to SD rats reduced glucose-stimulated insulin secretion from isolated pancreatic islets (Dhar *et al*., 2010). GLUT2 is the isoform of the facilitative glucose transporters in the pancreatic β cells. GLUT2 is translocated from a cytosolic pool to the plasma membrane in response to increased extracellular glucose. GLUT2 also acts as a glucose sensor and when the extracellular glucose concentration increases it sends a signal inside the cell to cause insulin secretion (Leturque *et al*., 2009). GLUT2 is reduced in animal models of T2DM (Efrat *et al*., 1994). The reduced secretion of insulin in the MG and MG+BSO groups of rats can be partly explained by significantly reduced membrane GLUT2 in the pancreas (Fig. 8-5B).

In type I diabetes there is β cell death whereas T2DM is characterized by gradual apoptosis of islet β cells which ultimately results in reduced insulin secretion (Pick *et al*., 1998; Robertson and Harmon, 2006; Koyama *et al*., 1998; Efanova *et al*., 1998). MG has been shown to induce apoptosis in other cell types such as neutrophils (Wang *et al*., 2007), Schwann cells (Fukunaga *et al*., 2004) and Jurkat leukemia T cells (Du *et al*., 2000). We found that MG and MG+BSO had induced DNA fragmentation and cause apoptosis in pancreatic

cells (Fig. 8-7A, B). Reduced islet number would also contribute to reduced pancreatic insulin content and insulin secretion (Figs. 8-4 and 8-5A) as observed in this study.

To further elucidate the role of MG in the metabolic and biochemical abnormalities described above, one group of rats was treated with MG + ALA. We have recently shown that ALA attenuates acute MG-induced glucose intolerance in SD rats (18). Thus, ALA has acute MG scavenging action besides being a known AGEs-breaker (Wolffenbuttel *et al*., 1998; Guo *et al*., 2009; Doggrell, 2001). The attenuation of chronic MG induced deleterious effects by ALA strongly supports the role of MG as an inducer of insulin resistance and β-cell dysfunction.

The deleterious effects of MG were not random since there were no significant changes in the levels of biomarkers such creatine kinase, an indicator of muscle damage, creatinine an indicator of kidney function, and ALT and AST, which are enzyme markers for liver damage (Table 8-1). The reason for increased levels of total cholesterol and triglycerides and reduced levels of HDL induced by MG and MG+BSO treatment (Table 8-1) are not known and require further investigations.

The results of this study suggest that chronic high dietary carbohydrate may cause daily abnormal elevations of MG levels, which leads to cumulative pathologic changes in the islet β cell insulin secreting machinery and in the insulin signaling pathway in tissues such as the skeletal muscle and adipose tissue. The severity and the time course of progression of these pathologic changes to T2DM may depend on the overall health of the individual, any underlying oxidative stress, GSH levels and antioxidant defences.

In summary, we have shown for the first time that chronic administration of MG to SD rats induces abnormalities in glucose homeostasis and insulin secretion. The direct

involvement of MG in these deleterious effects is supported by the GSH synthesis inhibitor BSO, which caused a further increase in MG levels when combined with MG treatment; and attenuation of the deleterious effects of MG by the MG scavenger ALA. Our results strongly implicate MG as the mediator of high glucose-induced insulin resistance and T2DM that is becoming a serious health problem in the Western world (Van dam *et al*., 2002; Weigensberg *et al*., 2009; Willett *et al*., 2002; Clark, 2009). Further studies may show that specific and safe MG scavengers could be useful in preventing high glucose-induced insulin resistance and T2DM.

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CHAPTER 9

DISCUSSION

LIMITATIONS

AND

CONCLUSIONS

General discussion

Elevated levels of MG have been reported in diabetic subjects (Wang *et al*., 2007; McLellan *et al*., 1994) and animals (Jia and Wu, 2007) but the findings have been associative so far. In order to prove a direct role of MG in the pathogenesis of diabetes mellitus, T2DM specifically, it is necessary to administer MG exogenously to normal animals, such as SD rats and perform a battery of tests to see if the biochemical and metabolic abnormalities found in T2DM can be reproduced. The rationale for undertaking this project was primarily based on several *in vitro* studies performed in our lab and by other investigators. In *in vitro* studies, treatment of cultured adipocytes, 3T3 cells (Jia *et al*., 2006) or L6 skeletal muscle cells (Ribolet-Chavey *et al*., 2006), with MG impaired insulin signalling. Similarly, incubation of insulin with MG induced modification of the insulin molecule and reduced its ability to induce glucose uptake in cultured 3T3-L1 adipocytes (Jia *et al*., 2006). Fructose-fed SD rats develop insulin resistance and have elevated plasma MG levels and reduced adipose tissue insulin-stimulated glucose uptake (Jia and Wu, 2007). These studies suggest a causative role of MG in inducing insulin resistance in insulin sensitive tissues such adipose tissue and skeletal muscle. However, there are no studies on any effects of MG on the pancreatic islet insulin secreting β cells. Thus, a comprehensive study was planned in normal SD rats to examine the effects of exogenous MG when given acutely as a single dose and when given chronically for 4 weeks.

The project started with an investigation of the relative contribution of some of the putative precursors of MG formation to MG generation in cultured A10 VSMCs. As discussed in on earlier chapter, aminoacetone, an intermediate of protein metabolism, was the most potent precursor in generating MG on an equimolar basis. After a 3 h incubation, aminoacetone produced a 7 fold increase in MG levels above the basal value followed by

fructose (3.9 fold) and D-glucose (3.5 fold) (Dhar *et al*., 2008). Acetol, an intermediate of fatty acid metabolism, was comparatively less potent than D-glucose, and produced a 2.8 fold increase in MG above the basal value. High fructose, glucose and aminoactone *via* formation of MG increased oxidative stress (Dhar *et al*., 2008). The study demonstrated that MG formation in VSMCs depends on the nature and concentration of the substrate. In order to validate the comparison with D-glucose it was necessary to use 25 mM of other substrates, despite the knowledge that such high concentrations for these other substrates have not been reported in humans or animals in the literature. Thus, while the practical significance of these is difficult to gauge without further studies, these findings draw attention to fructose as an important source of MG, in view of the fact that diet in Western countries tends to be high in fructose, especially in the form of corn syrup. The potency of aminoacetone may have significant implications in subjects with high dietary protein intake or bodily turnover.

Since measurement of MG levels in various biological samples forms the vital parameter for all projects in the lab, it was necessary to ensure a sensitive, reproducible and standard method of measurement of samples of plasma, organs/tissues and cultured cells. MG is measured by HPLC in most labs, including ours. A project was undertaken to test various sample preparation protocols for HPLC. Significant differences were observed in the amount of MG detected in a given sample when the sample preparation protocol was altered (Dhar *et al*., 2009). Protocols that yielded consistent and reproducible values in biological samples were defined and recommended for other users in the paper that was published (Dhar *et al*., 2008). Since many investigators around the world use HPLC to measure MG levels, this was a significant and very satisfying achievement for me.

Endothelial dysfunction is a feature if early stages of many pathological conditions and is a harbinger of severe pathology of disease which can develop. These conditions include diabetes mellitus, hypertension, atherosclerosis and many others. More attention is being devoted by the scientific community to identify the benefits of treating endothelial dysfunction in the hope of preventing more severe cardiovascular pathology later on.

Typically, endothelial dysfunction is taken as reduced endothelium-dependent relaxation. The role of eNOS as a key enzyme generating one of the main vasodilatory mediators, *viz*. NO, is the focus of many studies. Previous studies have reported that hyperglycemia induces endothelial dysfunction and the mechanism is commonly ascribed to an increase in oxidative stress (Potenza *et al.*, 2009, Triggle, 2008, Nishikawa *et al.*, 2000, Brownlee, 2001, Du *et al.*, 2001). It is not yet known how glucose itself triggers the increase in ROS production. Since, MG is a well known initiator of oxidative stress, and since it also happens to be a reactive metabolite of glucose, it provided a sound rationale for my investigation on the possible role of MG as the mediator, or one of the mediators, of hyperglycemia induced endothelial dysfunction. Since there have been no reports on the involvement of MG itself in endothelial dysfunction, the results of my study showing MG-induced eNOS-mediated dysfunction were novel and exciting. The results showed that incubation of endothelial cells from two different species, *viz*. human and rat, with high glucose for 3 or 24 h increases MG formation in a concentration and time dependent fashion. Incubation of HUVECs and RAECs with different concentrations of MG showed that 30 μM MG resulted in intracellular MG levels that were comparable to those generated in the cells incubated with 25 mM glucose. Both hyperglycemia and MG reduced activity of eNOS, reduced serine 1177 phosphorylation and reduced bradykinin-stimulated NO production.

Incubation of rat aortic rings with high glucose and MG reduced ACh induced relaxation precontracted with phenylepherine. The effect of MG and high glucose on aortic rings, HUVECs and RAECs was prevented by MG scavenger, aminoguanidine. The reduced NO production is possibly due to reduced eNOS activity without any change in eNOS protein. Reduced eNOS activity can be due to reduced BK-stimulated serine-1177 phosphorylation of eNOS caused by MG (30 μM) or glucose (25 mM). BK stimulation induces eNOS phosphorylation at Ser-1177 and increases NO production. MG and high glucose also decreased reduced GSH levels and GSH-reductase protein expression in HUVECs and the effect was prevented by MG scavenger, aminoguanidine. Our study shows for the first time that high glucose induced endothelial dysfunction is mediated by MG by reducing eNOS phosphorylation/activity, and NO bioavailability.

In order to undertake a systematic study on the effects of exogenous MG on glucose homeostasis *in vivo*, I started by examining the effects of acute administration of a single dose of MG to normal SD rats. Acute administration of MG significantly increased plasma MG levels and the levels were still higher at 2 h post MG administration as compared to control rats, suggesting a long half life of this dicarbonyl compound. Previous studies have shown MG impairs the insulin signalling *in vitro* by altering the structure and function of the insulin molecule (Jia *et al*., 2006). Interestingly, a single acute dose of MG to normal SD rats impaired glucose tolerance and induced insulin resistance (Dhar *et al*., 2010). After a bolus dose of glucose, the glucose tolerance was significantly impaired and insulin levels were higher in MG treated rats. To determine the possible reason of how MG induced insulin resistance, insulin stimulated glucose uptake was done in adipose tissue and insulin signalling pathway was studied. Our study showed that there was a significant decrease in insulin

stimulated glucose uptake, GLUT4 protein expression, IRS-1 tyrosine phosphorylation in adipose tissue of MG treated rats. To investigate whether MG induced effects can be prevented by MG scavenger, ALA, we gave a single acute dose of alagebrium in one group of rats 15 min prior to the administration of MG. ALA significantly attenuated the effects of MG, by scavenging it. We also observed significant increase in MG levels in aorta after acute administration. These data suggest that even a single acute dose of MG induces glucose intolerance and contributes to the development of insulin resistance in normal SD rats.

The next logical experiment was to examine the effects of chronic administration of MG to normal SD rats. The protocol for this study was designed as comprehensively as possible to investigate the effects of MG and the underlying molecular mechanisms in detail. MG was administered by continuous infusion with an implanted minipump for one month. To investigate whether endogenous inhibition of MG detoxification can exacerbate the effect of MG, one group of rats was treated with buthionine sulfoximine (BSO) in drinking water along with MG. BSO is a GSH synthesis inhibitor. GSH is one of the cofactors involved in the metabolism of MG to D-lactate *via* glyoxalase pathway. One group of rats was treated with the MG scavenger, ALA, along with MG. The MG levels were significantly elevated whereas GSH levels were significantly decreased in plasma, adipose tissue, skeletal muscle and pancreas of MG and MG+BSO treated rats. High plasma glucose levels and impaired glucose tolerance were observed in MG treated rats. An investigation of the mechanism of high plasma glucose indicated that insulin stimulated glucose uptake in adipose tissue and GLUT4 translocation to the plasma membrane were significantly reduced in MG treated rats. To investigate whether insulin sensitivity was affected, glucose stimulated insulin release from freshly isolated pancreatic islets was done in MG treated rats. Glucose stimulated insulin

release in islets of MG treated rats was significantly reduced. Moreover GLUT2 protein expression was significantly downregulated in MG and MG+BSO treated rats. Apoptosis of pancreatic β-cells was also observed in MG and MG+BSO treated rats.

Mechanism for MG induced T2DM and endothelial dysfunction

MG induces oxidative stress

Free radicals are molecules with an unpaired electron. ROS includes both oxygen derived free radicals and non radicals which do not have an unpaired electron in their orbit. An imbalance between oxidant and antioxidant state in the body creates a condition called oxidative stress. Oxidative stress is associated with number of diseases like diabetes mellitus, stroke, hypertension and atherosclerosis (Desai, *et al*., 2010). The most widely used markers of oxidative stress are measurement of superoxide, hydrogen peroxide and peroxynitrite in biological system. Superoxide is formed by the addition of an electron to the oxygen molecule and is mainly formed during mitochondrial electron transport chain reactions. Superoxide is cleaved by the enzyme superoxide dismutase into hydrogen peroxide and oxygen molecule (Desai, 2008). There are number of methods for the measurement of superoxide in biological samples. One of the most widely used is chemiluminescence based lucigenin assay using probe bis-*N*-methylacridinium nitrate (lucigenin). Increased levels of superoxide have been found in *in vivo* and *in vitro* conditions in association with increased MG, which suggest that MG directly induces superoxide generation (Desai, 2008). In 13 week old spontaneously hypertensive rats, increased levels of superoxide in aorta are associated with increased aortic MG levels (Wang, *et al*., 2005). Treatment of VSMCs with MG increased superoxide formation in a concentration and time dependent manner and the effect was prevented by the superoxide scavenger SOD (Wang, *et al*., 2006). Superoxide formation has also been shown to

increase significantly in neutrophils exposed to MG for 60 min. Although it is not a free radical, hydrogen peroxide is another highly reactive molecule. Catalase is the main enzyme involved in hydrogen peroxide removal. Hydrogen peroxide is measured using the probe 2',7'-dichlorofluorescin diacetate (DCFH-DA). Once inside the cell, DCFH-DA is converted to DCFH2 and then oxidised to the fluorescent product DCF. DCF is easily detected in cells using a fluorescent microscope or spectrofluorometer (Desai & Wu, 2007). When superoxide reacts with nitric oxide, it forms peroxunitrite, a reactive molecule with a short half life. Peroxynitrite levels can be measured in biological systems using dihydrodichlorofluorescein (H2DCF) or dihydrorhodamine 123 (DHR-123). Peroxynitrite oxidizes dihydrodichlorofluorescein and dihydrorhodamine to fluorescent dichlorofluorescein or rhodamine 123 which can be detected using spectrofluorometer (Mart´ın-Romero *et al*., 2004)

MG impairs insulin gene transcription

Insulin resistance is observed in disease conditions like diabetes and obesity. Chronic hyperglycemia associated with insulin resistance leads to various diabetic microvascular and microvascular complications. Chronic hyperglycemia leads to AGE formation by initiating reaction between reducing sugars and free amino groups on proteins. A large body of evidence suggests that harmful effects of hyperglycemia are due to the formation of oxoaldehydes, MG, glyoxal and 3-deoxyglucosone (Desai *et al*., 2010). Incubation of VSMCs with high glucose increases MG formation. Moreover, CEL, one of the AGEs was also significantly increased in VSMCs treated with high glucose (Dhar *et al*., 2008). MG levels have also been found to be elevated in plasma of diabetic patients which correlates with the degree of hyperglycemia (Wang *et al*., 2006). Treatment of L6 muscle cells with MG at a concentration of 5 mM alters

IRS-1 phosphorylation and impairs insulin signalling pathway (Riboulet-Chavey *et al*., 2006). MG alters the structure of insulin by modification of B-chain *in vitro* in a concentration dependent manner. The resulting MG-insulin adduct reduces glucose uptake in 3T3 adipocytes and impairs the function of insulin molecule (Jia, *et al*., 2006). Our present study has reported for the first time that MG administered by continuous infusion with a minipump for 28 days to Sprague-Dawley rats induces molecular abnormalities characteristic of type II diabetes mellitus. The elevation of plasma glucose levels is due to reduced insulin-stimulated glucose uptake in adipose tissue and plasma membrane GLUT-4 protein. We also observed pancreatic islet β-cell insulin secreting machinery was significantly impaired in MG and MG+BSO treated rats. Glucose-stimulated insulin secretion was reduced in freshly isolated pancreatic islets, GLUT2 protein level was decreased, pancreatic insulin content was lower. The protein as well as mRNA expression of insulin promoter transcriptional factors PDX-1 and Maf-A were significantly down-regulated whereas protein expression of C/EBPβ, a repressor of insulin gene transcription, was significantly up-regulated in MG and MG+BSO treated rats. The reduced insulin levels seen in the present study, can be due to impaired function of pancreatic islets. We also observed apoptosis and DNA fragmentation of pancreatic islets in MG and MG+BSO treated rats. The harmful effects of MG were attenuated by the MG scavenger and AGEs breaking compound, ALA. The insulin content in the pancreas was reduced in rats treated with MG and MG+BSO. The possible reason is due to reduced transcription of insulin synthesis as indicated by reduced levels of the insulin gene promoters PDX-1 and Maf-A induced by MG. PDX-1 is an essential regulator of pancreatic cell development. Inactivation of PDX-1 in the β-cells has been shown to impair β-cell function and cause diabetes. Maf-A , other transcription factor, activates insulin as well as glucagon

gene expression in the pancreatic islet β and α cells, respectively. The expression of PDX-1 and MafA are reduced in beta-cells of diabetes, resulting in decreased insulin synthesis and secretion, which clearly supports our findings of MG as a likely causative factor in T2DM. The present study indicates that diet high in carbohydrates causes daily abnormal elevations of MG levels, which leads to cumulative pathologic changes in the islet β cell insulin secreting machinery and in the insulin signalling pathway. The severity and the time course of progression of these pathologic changes to T2DM depends on the health of the individual, any underlying oxidative stress, GSH levels and antioxidant defences.

MG induces apoptosis

Apoptosis, also known as programmed cell death, is characterised by nuclear fragmentation, cell shrinkage, chromatin condensation and DNA fragmentation. Apoptosis differs from necrosis in that necrosis is due to cell injury. The most widely used method to check apoptosis is the TUNEL assay. TUNEL detects the DNA fragmentation which results from apoptosis. In T1DM, there is a β cell death whereas in case of T2DM as the disease progresses there is apoptosis of pancreatic islets which lead to reduced insulin secretion. MG has been shown to induce apoptosis in rat mesangial cells by activating p38 mitogen activated protein kinases (MAPK) (Liu *et al*., 2003). Incubation of primary rat retinal pericytes with 400 μM of MG for 6 h induces apoptosis (Kim *et al*., 2010). We have shown in our study that chronic treatment with MG and MG+BSO induce DNA fragmentation and apoptosis of pancreatic islets.

Limitations of the study

MG is produced in the body through many metabolic pathways. However, there is no report available on the relative amounts produced in different cell types and tissues, and also about the sources of MG that contribute to its basal plasma levels. It appears that the glyoxalase system and GSH very efficiently metabolize MG under physiological conditions and keep plasma MG levels below 10 μM as reported in most studies. Despite this fact, most in vitro studies have employed very high concentrations of MG ranging from 100 μM to 5 mM and more. Such high concentrations possibly do not have any pathophysiological relevance. One parameter we tried to observe in our doses and concentrations employed was the range of intracellular and plasma concentrations that we achieved, which was within the range reported in most studies. It is interesting in this regard that we observed significantly much higher basal as well as post exogenous-MG levels in aorta in SD rats. Whether more MG is produced basally in the aorta or whether it is deposited from the plasma is a big question that we did not address.

In our studies on cultured VSMCs reported in Chapter 4, we had to use a concentration of 25 mM of precursors such as aminoacetone and fructose, despite the fact that such high concentrations of these substrates have not been reported in the body. However, in order to compare these substrates with D-glucose, the most common precursor, it was necessary to use the same concentrations for all substrates. Levels of 25 mM plasma glucose are found in STZ-induced diabetes and sometimes in human subjects whose diabetes is not controlled.

In our studies on endothelial dysfunction it should be pointed out that we focused on the eNOS-NO system and endothelium-dependent relaxation. It can be argued that a lack of other vasodilatory mediators such as prostacyclin and EDHF, or an excess production of vasoconstrictors such as endothelin may also contribute to dysfunction. These are avenues for

future research. Similarly, the endothelium plays many roles besides regulation of vascular tone. Aspects of dysfunction related to these functions of the endothelium were not addressed in the present study due to obvious time restraints. These aspects require extensive further studies.

Another problem that is encountered in in vivo administration of MG is the route of administration. There have been no systematic reports on the oral absorption and pharmacokinetics of MG. We examined the oral administration of MG to SD rats and found that even after a high oral dose of 200 mg/kg given as an intragastric bolus by gavage, the absorption was limited and the peak level was less than one third of the level achieved with 100 mg/kg administered by the i.p. route. In such a case, administration of MG in drinking water would necessitate doses even higher than 200 mg/kg, which would still cast a doubt on how much is or will be absorbed since rats drink water by small sips at a time.

Another major problem on studies with MG is the lack of absolutely specific scavengers for MG. As described in the introduction, the most widely used scavenger, aminoguanidine, has other effects such as inhibition of histaminase and iNOS (Desai and Wu, 2007). Metformin when used as aMG scavenger (Lopez, *et al*., 1999). , while being safer, has actions on insulin-sensitive tissues. NAC, another scavenger, despite being used clinically, e.g. for acetaminophen overdose, has direct antioxidant properties. However, we tried different concentrations of aminoguanidine initially and selected the lowest effective dose/concentration. ALA was developed as an AGE-breaker (Coughlan, *et al*., 2007; Guo, *et al*., 2009; Wolffenbuttel, *et al*., 1998)., but we investigated and reported its acute effects as a preventive agent against MG (Dhar *et al*., 2010).

CONCLUSIONS

Our present study strongly implicates MG as one of the causative factors for the development of diabetes mellitus and endothelial dysfunction. Numerous studies have reported elevated levels of MG under diabetic and hypertensive conditions. But it was unclear whether MG is the cause or effect of these pathological conditions. We have shown in our study that incubation of VSMCs, RAECs and HUVECs with glucose increases MG formation in a concentration dependent manner. The increase in MG formation is associated with increased oxidative stress. Increase in MG levels in HUVECs induces endothelial dysfunction by reducing eNOS activity, NO bioavailability and phosphorylation of eNOS at ser-1177.

Acute MG administration to normal SD rats for 2 h induces glucose intolerance and impairs insulin signalling pathway by reducing tyrosine phosphorylation of IRS-1. We also observed reduced insulin stimulated glucose uptake and GLUT4 protein expression in adipose tissue of MG treated rats. Chronic MG administration by mini pump for 28 days induced pancreatic β- cell dysfunction. There was a significant increase in MG levels and decrease in GSH levels in plasma, pancreas, skeletal muscle and adipose tissue of MG and MG+BSO treated rats. Insulin stimulated glucose uptake and GLUT4 translocation was significantly impaired in adipose tissue of MG and MG+BSO treated rats. There was also reduced glucose stimulated insulin release in freshly isolated pancreatic islets in MG treated rats. Moreover we observed impaired insulin content and insulin gene transcription in pancreas of MG treated rats. The effects of MG were prevented by MG scavenger and AGE breaking compound alagebrium. All of these observations and results strongly implicate MG as one of the causative factors for the development of T2DM.

In conclusion, MG is one of the causative factors for the development of endothelial

dysfunction and T2DM. Development of specific MG scavengers will help prevent the progression of high carbohydrate induced diabetes and endothelial dysfunction.

SIGNIFICANCE OF THE STUDY

Diabetes is one of the leading causes of death next to cancer and heart diseases. Currently the number of people affected by diabetes globally is 285 million and the number is expected to reach 438 million by the year 2030. In Canada alone 3 million people have diabetes and the number will be 3.7 million by the year 2020. It is estimated that economic cost for the treatment of diabetes in Canada will be $16.9 billion by the year 2020. The incidence of T2DM is rising due to obesity, changing life style and aging.

The role of MG in the pathogenesis of diabetes has gained more attention in recent years. Exploring the role of MG in the pathogenesis of diabetes will provide new mechanistic links and novel upstream therapeutic targets for the treatment and management of diabetes mellitus.

FUTURE DIRECTIONS

To further clarify the role of MG in the pathogenesis of diabetes, hypertension and vascular remodelling, we are planning to carry out the following experiments in near future:

1. Treat SD rats with MG for one month, measure blood pressure and do functional studies using myograph and organ bath.
2. Use a specific MG scavengers like SMG08 to attenuate the MG-induced endothelial dysfunction and T2DM.

3. Investigate the molecular mechanism of how MG induces hypertension and vascular remodelling by determining the protein and mRNA expression of α1, AT1, renin and ACE in VSMCs, aorta and kidney.
4. Find specific MG inhibitors with minimal side effects and check their therapeutic effect in different diabetic animal models. This study will help explore alternative treatment for the management of diabetes and its complications.

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